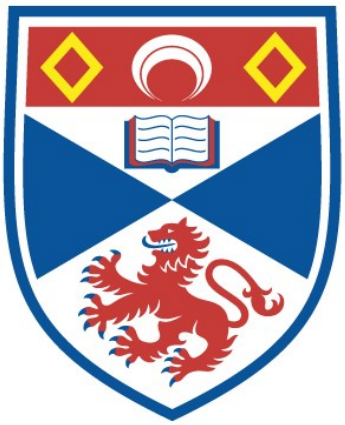
ATRIAL NATRIURETIC PEPTIDE RECEPTOR SUBTYPE DETERMINATION AND BIOLOGICAL ACTIONS OF ATRIAL NATRIUETIC PEPTIDED IN BOVINE CARDIAC MUSCLE AND HYPERTENSIVE RAT LIVER

Shirley McCartney

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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A thesis submitted to the University of St. Andrews for the degree of Ph.D.

by

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ABSTRACT

Atrial natriuretic peptide (ANP) has previously been shown to bind to specific ANP receptors and increase intracellular cGMP levels in purified rat cardiac sarcolemmal membranes. Experiments described in this thesis were performed to investigate the binding characteristics of ANP in bovine ventricular sarcolemmal membranes and in plasma membranes isolated from the liver of hypertension-resistant and hypertension-sensitive Dahl rats fed on two dietary salt regimes one of 0.8% NaCl and the other 8% NaCl. Additional experiments utilising ANP analogues in radio-receptor assays and radio-receptor crosslinking assays were performed to determine the precise nature of the ANP receptor population present in these membrane preparations.

In bovine ventricular cardiac sarcolemmal membranes, ANP bound specifically to one class of ANP receptor with a K_d of approximately 44 pM and a B_{max} of approximately 49 fmol/mg protein. ANP produced a 1.8-fold stimulation of manganese-dependent guanylate cyclase activity with an EC_{50} value of approximately 1 nM. Receptor binding using the des-ANP analogue indicated the predominant presence of the ANP-B receptor subtype. Radioreceptor crosslinking experiments did not entirely agree with these experiments. Radio-receptor crosslinking indicated the presence of two ANP receptors one of 60 kDa and one of 120 kDa, equivalent to the molecular weights of ANP receptors found in other tissues. Collectively these experiments indicate that bovine ventricular sarcolemmal membranes possess ANP receptors, at least a proportion of which are coupled to guanylate cyclase (ANP-B receptors).

In plasma membranes from the liver of Dahl-Resistant (Dahl-R) and Dahl-Sensitive (Dahl-S) rats, ANP bound specifically to one class of ANP receptor with K_d values ranging from 245 to 288 pM and B_{max} values ranging from 104 to 217 fmol/mg protein. ANP produced a 3.8 to 6.15-fold stimulation of manganese-dependent guanylate cyclase activity with an EC_{50} values ranging from 2.3 to 7.4 nM, dependent on the strain of Dahl rat and the dietary salt regime used. In liver membranes isolated from rats sensitive to salt-induced hypertension results indicated increases in B_{max} with no change in K_d for ANP binding to receptors and higher basal and ANP-stimulated guanylate cyclase levels. Receptor binding using the des-ANP analogue indicated the presence of 13-33% ANP-C receptors with a majority of ANP-B receptors in plasma membranes isolated from the liver of Dahl-R and Dahl-S rats. However, radio-receptor crosslinking experiments were unable to support these results. Collectively these experiments indicate that in plasma membranes isolated from the liver of Dahl-R and Dahl-S rats possess ANP receptors, at least a majority of which are coupled to guanylate cyclase (ANP-B receptors) and that sensitivity to hypertension induced by a high salt dietary regime increases the density of ANP receptors coupled to guanylate cyclase.

ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
cAMP	cyclic adenosine 5'-monophosphate
AM	arachnoid mater
ANF	atrial natriuretic factor
ANP	atrial natriuretic peptide
ANP-B	atrial natriuretic peptide B receptor
ANP-BR1	atrial natriuretic peptide B receptor 1
ANP-BR2	atrial natriuretic peptide B receptor 2
ANP-C	atrial natriuretic peptide C receptor
Ang II	angiotensin II
ATP	adenosine 5'-triphosphate
B_{max}	maximum binding
BNP	brain natriuretic peptide
BP	blood pressure
BS membranes	bovine ventricular sarcolemmal membranes
°C	degrees celcius
Ca^{2+}	calcium
CHAPS	3-[(3-cholamindopropyl) dimethylammonio]-1-propanosulfonate
CNP	C-type natriuretic peptide
CP	choroid plexus
cpm	counts per minute
DAG	diacylglycerol
des-ANP	des [QSGLG] ANP (5-23)-NH_2
DFDNB	1,5-difluoro-2,4-dinitrobenzene
DNA	deoxyribonucleic acid
cDNA	complementary deoxyribonucleic acid

DOCA	deoxycorticosterone acetate
DSS	disuccinimidyl suberate
EC_{50}	concentration required for half maximal stimulation
EDRF	endothelium derived relaxant factor
EDTA	ethylenediamine tetracetic acid
EGS	ethylene glycol*bis*(succinimidylsuccinate)
GTP	guanosine 5'-triphosphate
cGMP	cyclic guanosine 5'-monophosphate
Hepes	N-2-hydroxyethylpiperazine-N'-ethanosulfonic acid
HSAB	N-hydroxysuccinimidyl-4-azidobezoate
^{125}I-Az-Bz-ANP	^{125}I-azidobezoate photoaffinity derivative of ANP
IC_{50}	concentration required for half maximal inhibition
iso-ANP	iso-rat atrial natriuretic peptide
IP_3	inositol 1,4,5-triphosphate
1K-1C	one kidney-one clip
2K-1C	two kidney-one clip
KCl	potassium chloride
K_d	dissociation constant
kDa	kilo-daltons
min	minutes
mg	milligrams
µg	micrograms
Na	sodium
NHS	N-hydroxysuccinimide
NSB	non-specific binding
OB	olfactory bulb
PDGF	platelet derived growth factor
PEI	poltethyleneimine
Phos'don	phosphoramidon

PI	phosphatidyl-inositol
poly $(A)^+$ RNA	3'-polyadenylated ribonucleic acid
PMSF	phenylmethylsulphonyl fluoride
mRNA	messenger ribonucleic acid
rpm	revolutions per minute
S.D.	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
S.E.M.	standard error of the mean
SFO	subfornical organ
SHR	spontaneously hypertensive rat
TPA	12,-*O*-tetradecaonylphorbol-13-acetate
Tris	[2-amino-2-(hydroxymethyl) propane-1,3-diol (tris)]
Tyr^8-ANP	[Tyr^8] (rat)-atrial natriuretic factor (5-27)
TX-100	triton-X-100
WKY	Wistar-Kyoto

FIGURES AND TABLES

Table 4.5

Comparison of the concentration required for half maximal stimulation (EC_{50}) of cGMP production and the K_d values for [^{125}I]-ANP specific binding.

EC_{50} values were calculated for cGMP production and [^{125}I]-ANP specific binding in liver membranes isolated from (a) Dahl-R rats on a 0.8% NaCl diet, (b) Dahl-R rats on an 8% NaCl diet, (c) Dahl-S rats on a 0.8% NaCl diet and (d) Dahl-S rats on an 8% NaCl diet.

RAT GROUP	EC_{50}cGMP (nM)	K_d (pM)
a	3.16 ± 2.04	245 ± 80
b	2.33 ± 0.47	258 ± 18
c	2.9 ± 0.71	285 ± 32
d	7.33 ± 2.05* +	288 ± 84

* significantly different from the EC_{50} value of Dahl-S rats on a 0.8% NaCl diet ($p \leq 0.05$ mean of 3 experiments ± SD)

+ significantly different from the EC_{50} value of Dahl-R rats on an 8% NaCl diet ($p \leq 0.05$ mean of 3 experiments ± SD)

CHAPTER 1

INTRODUCTION

1.1 ATRIAL NATRIURETIC PEPTIDE

1.1.1 History of ANP

Atrial natriuretic factor (ANF) is stored primarily in specific granules of atrial myocytes and plays a key modulatory role in the regulation of extracellular fluid volume and blood pressure, (DeBold *et al.* 1981). The presence of specific granules, in the atria, was first described by Kisch in (1956). In the same year, Henry and co-workers (Henry *et al.* 1956), reported that distension of the left atrium altered urinary excretion, however these two separate observations were not linked for over two decades. For many years the atrial specific granules (Jamieson & Palade 1964) were thought to serve as storage sites for endogenous catecholamines in the heart, (Palade 1961; De Bold & Bencosme 1973). By the seventies Marie *et al.* (1976) and De Bold (1979), demonstrated that water loading and increasing sodium levels caused a significant decrease in the number of atrial cell granules. In 1981, De Bold *et al.* (1981) demonstrated that injection of atrial, but not ventricular tissue extracts, into anaesthetised rats caused a marked increase in urinary sodium and water excretion. The substance in these extracts was given the name atrial natriuretic factor (ANF). Confirmation that these atrial specific granules were the likely storage sites for ANF came in 1982, with the demonstration that partially purified granules showed natriuretic and diuretic properties (De Bold 1982; Garcia *et al.* 1982).

De Bold's work led to the characterisation and sequencing of a closely related family of peptides from the atria, (see section 1.1.4). They were given various names; atriopeptins, auriculins, cardionatrins and ANF's. ANF is now known to be a peptide and is generally referred to as Atrial Natriuretic Peptide (ANP).

Since its discovery almost 10 years ago, many studies have been carried out on the biochemistry and physiology of ANP both *in vitro* and *in vivo*. The information obtained from these studies is summarised in the next sections and includes a detailed description of the synthesis and structure of ANP, ANP receptors and endogenous guanylate cyclase activity, mechanisms controlling the release of ANP, some physiological and pharmacological actions of ANP and the role played by ANP in hypertension.

1.1.2 ANP Gene Structure

Using complementary DNA (cDNA) probes specific for ANP, the genomic DNA encoding prepro-ANP has been identified in man, (Greenberg *et al.* 1984; Nemer *et al.* 1984) mouse, (Seidman *et al.* 1984) rat, (Argentin *et al.* 1985) and ox, (Vlasuk *et al.* 1986). The gene sequence is highly conserved between species and possibly exists as a single copy, (Seidman *et al.* 1984; Argentin *et al.* 1985; Oikawa *et al.* 1984). Nucleotide sequence analysis of these genes shows that human, rat, mouse and ox have 3 coding regions (exons) and 2 intervening sequences (introns). The first exon encodes an untranslated 5' sequence, the ATG initiation codon, a signal peptide sequence of 24 amino acids and the first 17 amino acids of proANP. The second exon encodes the remaining amino acid sequence of proANP with the exception of 1 amino acid in human and 3 amino acids in rat, mouse

and ox. These C-terminal amino acids are encoded in the third exon which also contains the stop codon and an untranslated 3' sequence, (see fig. 1.1).

The second intron of the rat and the human ANP gene contains a consensus sequence for a potential glucocorticoid receptor binding site, (Greenberg *et al.* 1984; Argentin *et al.* 1985). This raises the possibility that ANP gene expression may be regulated by glucocorticoids. Gardner *et al.* (1986, 1988) and Nemer *et al.* (1987) support this with evidence that glucocorticoids produce a 2-3 fold increase in atrial ANP-specific mRNA levels in the rat.

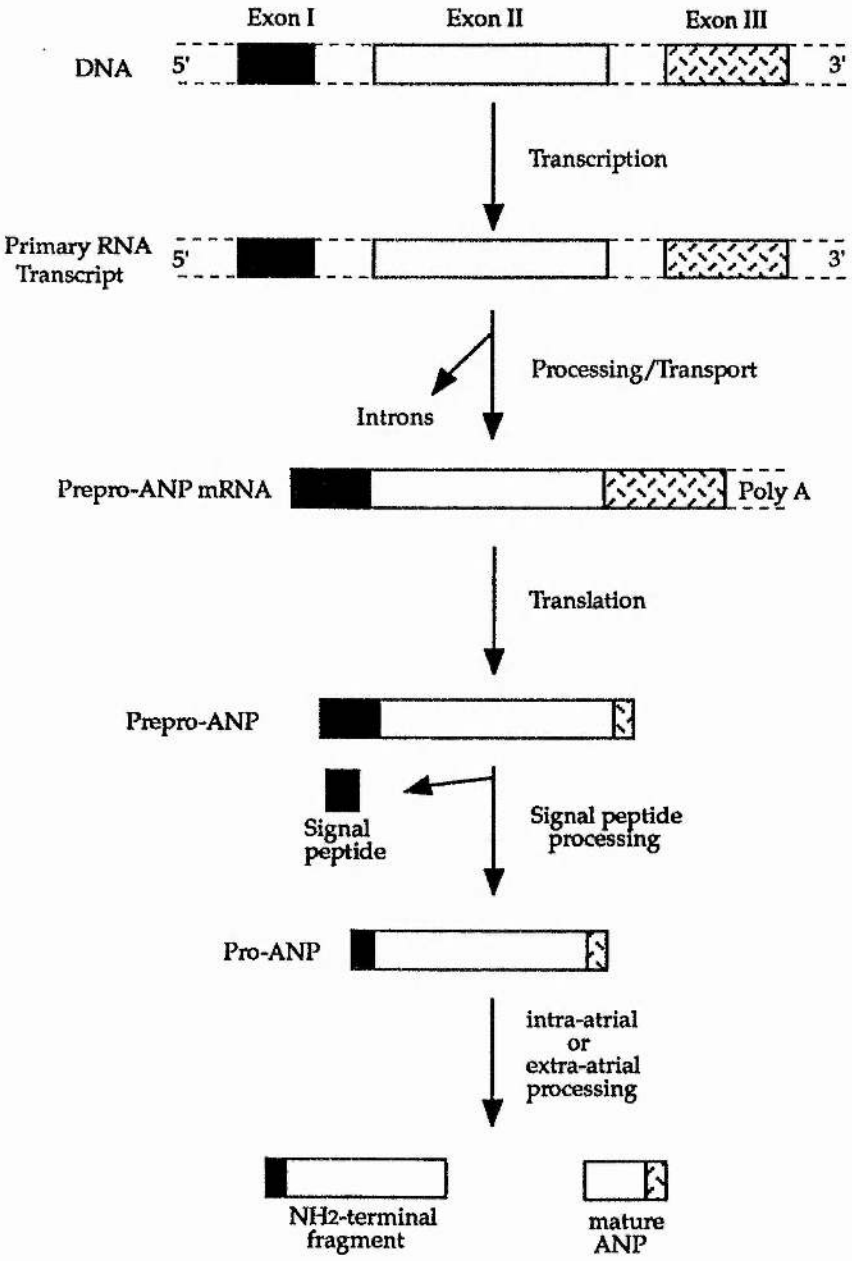
1.1.3 Gene Expression of ANP

1.1.3.1 ANP Gene Expression in the Heart

In atrial tissues, ANP-specific mRNA represents approximately 1-3% of the total poly(A)$^+$ RNA, (Bloch *et al.* 1987). Nakayama *et al.* (1984) studied the regulation of gene transcription and showed that ANP mRNA levels in water deprived rats decreased by approximately 50% after 2 days dehydration and by 75% after 4 days dehydration, the latter was in conjunction with a 60% depletion of total atrial mRNA. Takayanagi *et al.* (1985) also observed 70% decreases in the level of ANP-specific mRNA in water deprived animals (5 days) with a concurrent 50% decrease in plasma ANP concentration and a 200% increase in atrial ANP content. This was in agreement with Marie *et al.* (1976) and De Bold *et al.* (1979), who demonstrated that induction of water deprivation and sodium deficiency caused significant increases in the number of atrial granules. Takayanagi *et al.* (1985) reported that ANP-specific mRNA concentrations decreased in rats on a low salt diet, but that the concentrations in animals

Figure 1.1

Structure of the Atrial Natriuretic Peptide Gene



on a high salt diet did not differ from the control animals. Recently however, Hong *et al.* (1990) showed that short term water deprivation in rats for 2 and 4 days caused an increase in ANP-specific mRNA by 2.1 and 1.6-fold respectively, with a slight decrease at 6 days of 0.38-fold. They also reported increased ANP-specific mRNA gene expression with short term, salt loading (0.9% NaCl in drinking water) for 2, 4 and 6 days with 2.4, 2.8 and 2-fold increases respectively. These results all suggest that body fluid and sodium balance are associated with ANP gene expression in the heart, although there still exists some controversy as to the precise nature of this relationship.

1.1.3.2 ANP Gene Expression in other tissues

Although the major source of plasma ANP is the secretory vesicles of the atria, cDNA probes have identified extra-atrial expression of ANP (Gardner *et al.* 1985). ANP-specific mRNA has been detected in the ventricles, lung, pituitary gland and the hypothalamus of the rat, although in concentrations 100-250 fold less than those found in atria. Expression of the ANP gene in brain (Morii *et al.* 1985; Saper *et al.* 1985), lung (Sakamoto *et al.* 1985b) and kidney (Sakamoto *et al.* 1985a), has also been demonstrated by both immunohistochemical and biochemical analayses. The precise physiological function of ANP in these extra-atrial tissues remains unknown. Gutkowska & Nemer (1989) provides a full summary of structure, expression and function of ANP in extra-atrial tissues. Gardner *et al.* (1987), detected ANP-specific mRNA in distal thoracic aorta at levels less than the aortic arch which were in turn much less than that found in the atria. They also visualised ANP using immunochemistry in these tissues. Gardner *et al.* (1987) speculated that ANP may play an important role as a paracrine/autocrine factor. Gardner

et al. (1985), Nemer *et al.* (1986) and Takayanagi *et al.* (1987a) suggested that the presence of ANP containing cells in the ventricle may allow for secretion of ANP into the circulation, in addition to ANP secretion from the atria. Interestingly, the levels of ANP and ANP-specific mRNA in the ventricles can be altered under certain conditions. Takayanagi *et al.* (1987a) observed ventricular ANP levels 3-fold higher in spontaneously hypertensive rats compared to normotensive controls. Lattion *et al.* (1986) and Nemer *et al.* (1987) showed a preferential increase in ventricular ANP-specific mRNA (4 to 11-fold) over atrial ANP-specific mRNA (1.5 to 3.3-fold) with volume overload and glucocorticoid administration.

1.1.4 Secretion and Processing of ANP

The amino acid sequences of a number of rat atrial peptides possessing natriuretic and diuretic properties were published simultaneously by various laboratories; (Flynn *et al.* 1983; Currie *et al.* 1984a; Geller *et al.* 1984; Misono *et al.* 1984a; Misono *et al.* 1984b; Seidah *et al* 1984; Kangawa *et al.* 1985a; Kangawa *et al.* 1985b and Atlas *et al.* 1984). These reports indicated the presence of a variety of peptides ranging from 19-35 amino acids in length. These initial studies indicated significant similarities between the different peptides. Kangawa & Matsuo (1984) and Thibault *et al.* (1984) carried out similar work with human atrial extracts and isolated an ANP sequence which only differed from rat atrial ANP sequences in possessing a methionine residue in place of an isoleucine residue at position 12, of the mature peptide (see fig. 1.2). Essentially all of the peptides isolated were extended or truncated versions of the same peptide, which contained a basic 17 amino acid intramolecular sulphide loop sequence. Larger molecular weight peptides were shown to be

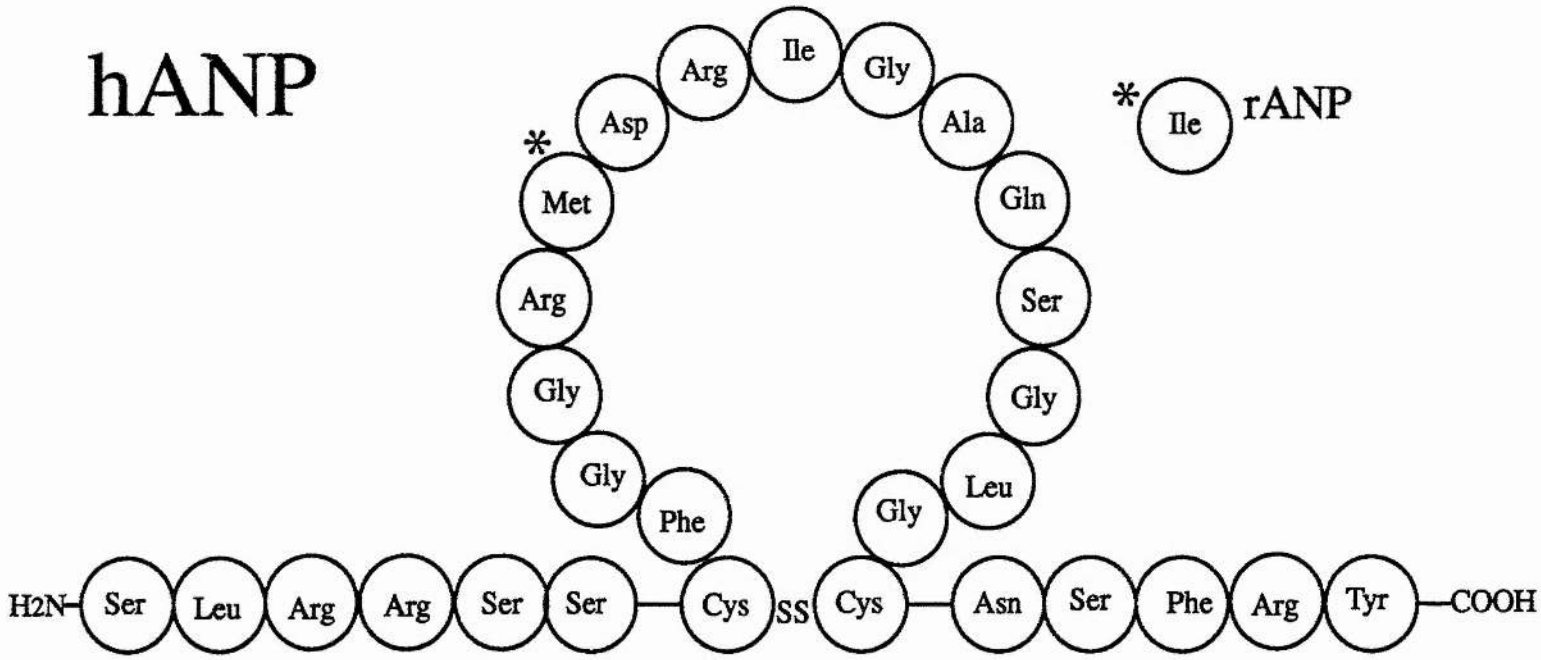


Figure 1.2

Amino Acid sequence of Human and Rat Atrial Natriuretic Peptide (ANP)

quickly converted to low molecular weight forms. Trippodo *et al.* (1984b), showed this by mixing higher molecular weight forms with crude atrial extracts. Currie *et al.* (1984b) also showed this by gentle proteolysis of higher molecular weight forms. These observations strongly supported the idea that low molecular weight forms appeared to be derived from the carboxy-terminal of a higher molecular weight precursor protein.

The primary structure of the ANP precursor was determined from the DNA sequence of ANP cDNA clones by location of the reading frame containing the C-terminal ANP peptides and following its N-terminus to the ATG initiation codon and a classic signal peptide sequence, (Perlman & Halverson 1983). (Most secretory proteins possess an N-terminal sequence of 16-30 hydrophobic amino acids (the signal peptide sequence) that is thought to initiate transport across the endoplasmic reticulum (Darnell *et al.* 1986)). Translation of a prepro-ANP cDNA clone in the rat revealed a 152 amino acid sequence of preproANP (Maki *et al.* 1984), of which the first 24 amino acids are the signal sequence. In the human prepro-ANP was found to be 151 amino acids in length (Oikawa *et al.* 1984), of which the first 25 residues are the signal sequence. ANP is the final 28 amino acids, (known as α-ANP) at the carboxy-terminal of the prepro-ANP sequence. Human prepro-ANP shares 80% homology with the rat peptide, with the greatest similarity at the carboxy-terminal of ANP, where there is only one amino acid of a difference.

Pro-ANP, (known as γ-ANP) in the rat, formed after signal peptide processing and removal of the COOH-terminal arginine dipeptide of rat preproANP, is 126 amino acids in length. Pro-ANP in the human, formed after signal peptide processing of human preproANP is also 126

amino acids in length. A 30 amino acid N-terminus sequence of pro-ANP is homologous to the N-terminus sequence of a peptide with vasodilatory properties, cardiodilatin, previously extracted from porcine atria (Frossman *et al* 1983; 1984).

In plasma, α-ANP has been identified (Miyata *et al* 1985) as the main circulatory form (Glembotski *et al.* 1985; Imada *et al.* 1985), whereas in the human atrium, the main storage form has been identified as γ-ANP, although this form has also been found in low concentrations in the circulation (Kangawa *et al.* 1985). The rat atrium also contains γ-ANP as its major storage form (Kangawa *et al.* 1984). Conversion of the high molecular weight precursor to the 28 amino acid active peptide either takes place immediately before, or more likely immediately after secretion from the atrial cardiocytes. The mechanism of this conversion has not yet been elucidated. However a specific extracellular atrial protease has been implicated in the final processing (Rugg *et al.* 1988; Johnson & Foster 1990).

Another circulatory related but distinct form of ANP has been identified in human plasma, known as β-ANP (56 amino acids) (Kangawa *et al.* 1984; 1985). β-ANP is an antiparallel dimeric form of α-ANP (Kangawa & Matsuo 1984; Nakao *et al.* 1984). Kangawa *et al.* (1985) showed that α-ANP did not undergo dimerisation to β-ANP with the experimental purification conditions utilised. In addition, Kangawa *et al.* (1984) observed that β-ANP was endogenous to the atria although the process in which dimerisation takes place *in vivo* remains unknown. Itoh *et al.* (1988) reported that β-ANP is converted to α-ANP in the human

circulation. The natriuretic and diuretic properties of β-ANP are slower acting than α-ANP, though β-ANP has a more potent and lasting action.

The presence of both γ-ANP and α-ANP have been reported in rat ventricle, with α-ANP, (Inagami *et al.* 1987) and γ-ANP (Miyata *et al.* 1986) being reported as the predominant form, respectively. Tanaka *et al.* (1984) showed that ANP in the brain consists mainly of α-ANP and an intermediate form (less than 28 but more than 25 amino acids). Imada *et al.* (1985) also reported the existence of several ANP like peptides of 28 amino acids or less in the brain. This finding has been supported by other workers including, Morii *et al.* (1985), Glembotski *et al.* (1985) and Shiono *et al.* (1986).

1.1.5 Other related Natriuretic Peptides

In addition to β-ANP and γ-ANP, (see section 1.1.4) a related peptide, initially isolated from porcine brain (Sudoh *et al.* 1988a; 1988b) and then from porcine heart (Kambayaski *et al.* 1990) has been shown to possess natriuretic and diuretic properties similar to that of ANP. This peptide has been named, brain natriuretic peptide (BNP). BNP comprises 26 amino acids and shares 60% homology with ANP. Iso-rat atrial natriuretic peptide, (iso-rANP), another related natriuretic peptide has been identified from rat atria (Flynn *et al.* 1989; Jennings & Flynn 1989 and 1990). Iso-rat ANP is composed of 45 amino acids and its concentration in the rat atria is less than 1% of that of ANP (Flynn *et al.* 1989). Homology between ANP, BNP and iso-rANP resides mainly within the loop of amino acids formed by the intra-disulphide bond, with less homology at the amino and carboxy-termini. More recently another peptide known as CNP (C-type natriuretic peptide) has been isolated from

porcine brain, (Sudoh *et al.* 1990). CNP has an amino acid sequence homologous to both ANP and BNP and also contains an intramolecular disulphide bond. It is however unique in having 5 amino acids at the N-terminal and possesses no C-terminal, (see fig. 1.3). Kambayaski *et al.* (1990) showed that the sequence of BNP is species specific i.e. the specificity of BNP is highly conserved within a species but not between species. This is in direct contrast to both ANP and CNP (Arimura *et al* 1991) where amino acid sequences are highly conserved between species. Most experimental data would seem to suggest that there exists an extended family of natriuretic and diuretic peptides all similar in structure, with related ligand-receptor action.

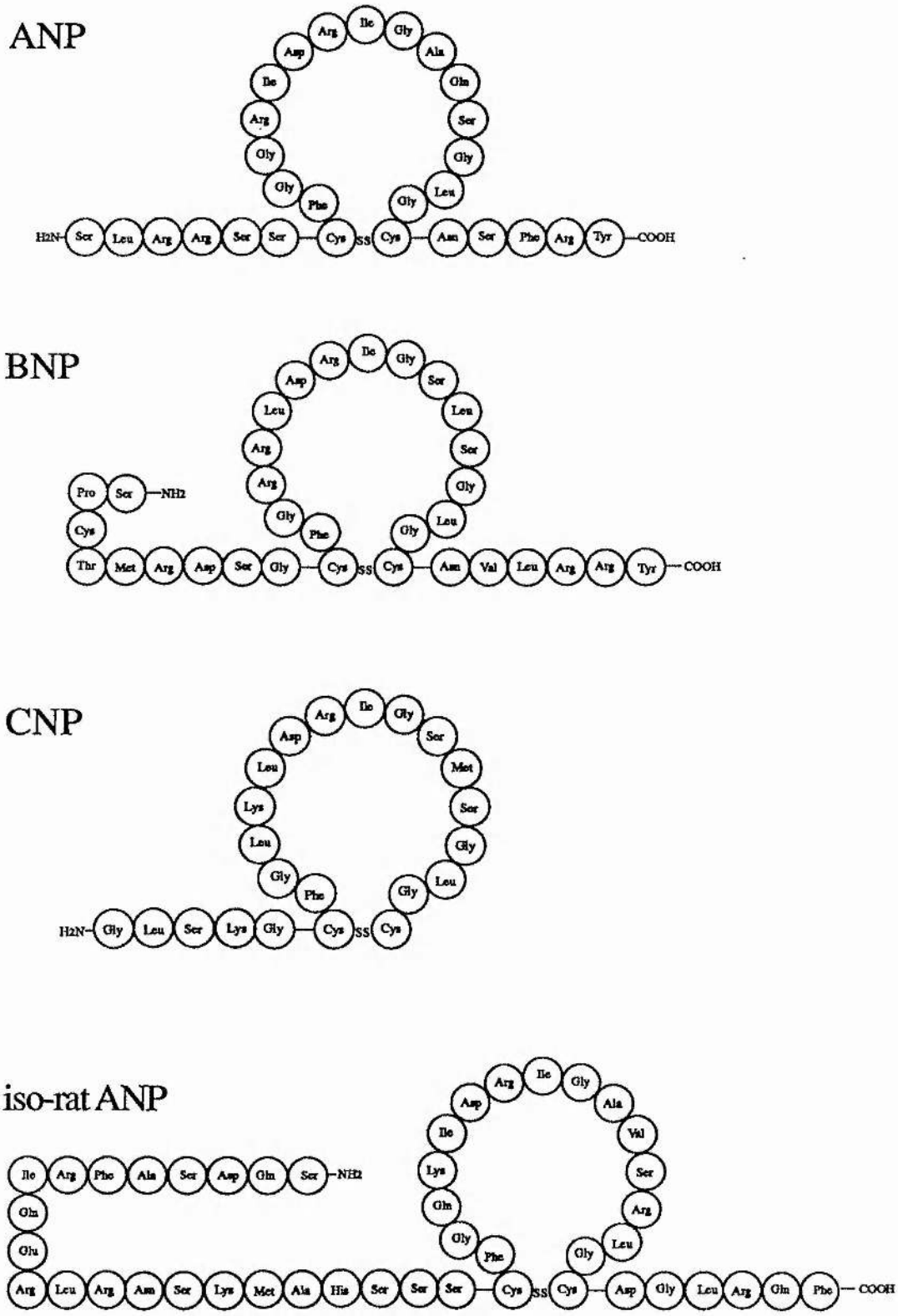
1.2 ATRIAL NATRIURETIC PEPTIDE RECEPTORS

1.2.1 Localisation of ANP receptors

The physiological effects of ANP are mediated via interaction with specific receptors in various target tissues. The determination of the amino acid sequence of ANP and the use of radiolabelled ANP ligands has led to the identification and characterisation of ANP receptors. Specific ANP receptors have been identified in a wide variety of tissues, including kidney (Koseki *et al.* 1985), adrenal cortex (Meloche *et al.* 1986a; 1986b), brain (Lynch *et al.* 1986) and lung (Olins *et al.* 1988). Specific ANP receptors have also been found in almost all animals so far studied, including rat (Rugg *et al.* 1989), rabbit (Olins *et al.* 1988), ox (McCartney *et al.* 1990) and human (Schiffrin *et al.* 1988). The ANP receptor is thus ubiquitous in vertebrates. Details of these ANP receptors are summarised in the following section.

Figure 1.3

Amino acid sequences of porcine ANP, BNP, CNP and iso-rat ANP



1.2.2 Characterisation of ANP receptors

Many aspects of ANP-receptor interaction have been investigated in several tissues in an attempt to identify and characterise receptors in terms of their molecular size and type present and their respective second messenger systems. In the absence of completely pure ANP receptor protein, affinity labelling of ANP receptors with bifunctional cross-linking agents and photolabile analogues of ANP followed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography has provided much information on the molecular size of these receptors. Vandlen *et al.* (1985) used a photoaffinity azidobenzoate derivative of ANP (^{125}I-AzBz-ANP) to bind to rabbit aortic membranes. Analysis after exposure to ultra-violet light and SDS-PAGE under reducing conditions (i.e. in the presence of β-mercaptoethanol) revealed three distinct polypeptides of 120, 70 and 60 kDa. The same three polypeptides were labelled, with the 60 kDa component being most intense, when the chemical cross-linkers disuccinymidyl suberate (DSS) and 1,5-difluoro-2,4-dinitrobenzene (DFDNB) were used. In the same year Yip *et al.* (1985) utilised a photoaffinity *N*-hydroxysuccinimide ester derivative of ANP (^{125}I-iodoazidosalicylic acid-ANP) in binding studies with rat kidney cortex plasma membranes. This group identified a 140 kDa glycoprotein in rat kidney cortex plasma membranes and a 140 kDa protein was also specifically labelled in rat liver plasma membranes but not in rat adipose tissue. Hirose *et al.* (1985) solublised bovine adrenal cortex receptors with 3-[(3-cholamidopropyl) dimethylammonio]-1-propanosulfonate (CHAPS) before chemically cross-linking with DSS. They observed an ANP receptor protein of 130-140 kDa after SDS-PAGE under non-reducing conditions (i.e. in the absence of β-mercaptoethanol) and a 70 kDa ANP receptor protein with SDS-PAGE under reducing

conditions. The evidence of this group suggested that the native ANP receptor protein is composed of at least two subunits which together form a disulphide-linked 130-140 kDa unit capable of binding ANP.

The information obtained from these initial crosslinking experiments indicated the presence of at least three specific ANP receptors, one of approximately 60 kDa, one of approximately 120 kDa composed of two 60 kDa subunits and one of approximately 120 kDa.

To further clarify ANP receptor molecular size, type and function, purification of the receptors was also attempted. Takayanagi *et al.* (1987a) and (1987b) purified two receptor types from bovine adrenal zona glomerulosa cell membrane fractions, one with, and one without endogenous guanylate cyclase activity. Both receptors showed high affinity binding to ANP, however the co-purified guanylate cyclase-linked receptor showed a lower affinity to $ANP_{(103-123)}$ and $ANP_{(105-121)}$ analogues, (these analogues lack the C-terminal phenylalanine-arginine residues and numbering refers to the amino acid position of proANP) than the receptor without guanylate cyclase activity. Using the photoaffinity derivative, 4-azidobenzoyl-^{125}I-ANP and with SDS-PAGE under non-reducing conditions, both receptor types migrated as a single 135 kDa band. However, the presence of β-mercaptoethanol and SDS-PAGE revealed that the receptor without guanylate cyclase activity migrated as a 62 kDa band, whereas the co-purified guanylate cyclase-linked receptor remained at 135 kDa. Peptide map analyses of the primary structures of the two purified receptor subtypes from bovine adrenal zona glomerulosa cell membrane fractions was performed. Takayanagi *et al.* (1987a) found that more than 90% of the peptides generated from the two

receptors were different. Kuno *et al.* (1986b) supported the observation that ANP binding and guanylate cyclase activity occupied the same receptor protein. This group purified from rat lung, a 120 kDa protein possessing both ANP binding ability and guanylate cyclase activity.

In addition to using bifunctional cross-linking agents and photolabile analogues to determine the size of ANP receptors, structural analogues of ANP which bind with differing affinities and to different receptor types have also successfully provided information on ANP receptor type. In 1985, Garcia *et al.* (1985c) prepared a series of analogues produced by N-terminal chemical cleavage of synthetic $ANP_{(101-126)}$ yielding $ANP_{(102-126)}$, $ANP_{(103-126)}$, $ANP_{(104-126)}$ and $ANP_{(105-126)}$ or C-terminal enzymatic digestion yielding ($ANP_{(101-121)}$, $ANP_{(101-123)}$, $ANP_{(101-124)}$ and $ANP_{(101-125)}$. This group then examined the effects of these analogues and the additional synthetic analogues $ANP_{(103-123)}$, $ANP_{(103-125)}$, $ANP_{(96-126)}$ and $ANP_{(54-126)}$ on the inhibition of noradrenalin-induced contraction of rabbit thoracic aorta. They observed $ANP_{(101-126)}$ to be the most potent with the N-terminally cleaved analogues also producing a marked inhibition of the noradrenalin response. In contrast, removal of amino acids from the C-terminal markedly decreased the inhibitory effect of $ANP_{(101-126)}$. This observation indicated the importance of the ANP C-terminal in overcoming noradrenalin-induced contraction of rabbit thoracic aorta.

In the same year, Ballerman *et al.* (1985) showed that the affinity for ANP receptor binding in several cells and tissues was 10-1000 fold higher than the concentration of ANP required for half maximal stimulation of cGMP production. In support of this observation, Leitman & Murad (1986) and

Scarborough *et al.* (1986) investigated the relationship between ANP analogue binding and stimulated cGMP production in cultured bovine aortic endothelial and smooth muscle cells. These two groups showed that ANP analogues which were lacking in the carboxyl-terminal phenylalanine-arginine-tyrosine sequence retained a high affinity for nearly all vascular ANP receptors. However these ANP analogues were unable to stimulate cGMP generation or to antagonise the action of ANP stimulated cGMP generation. This observation was also supported by Leitman *et al.* (1986) who identified 130 and 66 kDa polypeptides in bovine aorta cultured endothelial cells, using the chemical cross-linker DSS. Leitman *et al.* (1986) showed that 0.1μM tyrosine-atriopeptin I ([Tyr^4]-ANP(4-25) a truncated ANP analogue lacking the carboxyl-terminal phenylalanine-arginine residues inhibited [^{125}I]-ANP binding to the 66 kDa site to a similar degree as ANP however, was 150-fold less able to inhibit binding to the 130 kDa site. In addition [Tyr^4]-ANP(4-25) was found only to produce a 4-fold increase in cGMP levels compared to a 400-fold increase produced with ANP. Leitman *et al.* (1986) speculated that the 130 kDa site was most likely coupled to a guanylate cyclase which was responsible for cGMP formation and that the carboxyl-terminal phenylalanine-arginine residues were important for linking the ANP receptor to the activation of guanylate cyclase. They also suggested that it was likely that the 66 kDa site was not coupled to guanylate cyclase. Lewicki *et al.* (1988) synthesised a series of ANP analogues which had systematic single amino acid substitutions at each position in the 17 amino acid intramolecular sulphide loop structure or deletions of one to five amino acids from the carboxy-terminal end of the looped structure. Amino acid deletions from within the loop sequence of ANP resulted in 100-1000 fold decreases in cGMP accumulation. However these same

amino acid deletions had no effect on the binding affinity to ANP receptor sites not coupled to guanylate cyclase. Their investigations confirmed previous studies in cells and tissues and showed there were two ANP receptor populations in cultured bovine aortic smooth muscle cells.

The biochemical and pharmacological studies outlined above have led to the identification of at least two distinct ANP receptor subpopulations. One subpopulation of biologically active receptors termed ANP-B receptors has a molecular weight of approximately 130 kDa and is coupled to guanylate cyclase. The ANP-B receptor requires carboxyl-terminal phenylalanine-arginine residues for high affinity binding. Activation of ANP-B receptors by ANP results in the stimulation of cGMP production.

The other receptor subpopulaton is thought to be "clearance" receptors (Maack *et al.* 1987). That is, receptors responsible for the binding and removal of ANP from the circulation and have been termed ANP-C receptors. These have a molecular weight of approximately 60 kDa and are able to bind various truncated ANP analogues with an almost equal affinity as ANP. Therefore, unlike the ANP-B receptor the ANP-C receptor does not require carboxyl-terminal phenylalanine-arginine residues for high affinity binding. ANP binding does not stimulate the production of cGMP with this receptor population.

1.2.3 Function of the ANP-B receptor and guanylate cyclase

Guanylate cyclase has been identified in virtually all cell types examined. It is the enzyme which catalyses the formation of cGMP from guanosine triphosphate (GTP). In most cells this enzyme exists as a polymorphic

protein and its activity can be attributed to the coexistence of cytostolic (soluble) and membrane-associated (particulate) forms. These soluble and particulate forms differ greatly in their physical and biochemical functions and characteristics. The concentrations of each of these forms within the cell varies with cell type, physiological state and experimental protocol used to assay the enzyme. In the adult liver (Kimura & Murad 1975a) or in blood platelets (Bohme *et al.* 1974) the soluble cytostolic form is predominant. In contrast, the particulate form predominates in regenerating and fetal liver (Kimura & Murad 1975b), intestinal mucosa (De Jonge 1975; Quill & Weiser 1975), retinal rod outer segments (Fleishman *et al.* 1980; Goridis *et al.* 1973; Krishanan *et al.* 1978) and in hepatomas and renal tumours (Criss *et al.* 1976; De Rubertis & Craven 1977; Goridis *et al.* 1977). In addition some cell types including C_6 rat glioma, B103 rat neuroblastoma (Sinacore *et al.* 1983) and sea urchin sperm (Gray & Drummond 1976; Garbers 1976; Radnay *et al.* 1983) exclusively possess the particulate form.

Soluble guanylate cyclase has been purified to apparent homogeneity from several sources and can be distinguished from the particulate enzyme by its sensitivity to exogenous nitro-vasodilators, such as nitroprusside, nitroglycerine, azides, nitrile and the endogenous vasorelaxant nitric oxide, (endothelial-derived relaxant factor (EDRF)). This soluble guanylate cyclase enzyme has an apparent molecular weight of 150,000 Da (Garbers 1979; Lewicki *et al.* 1980; Gerzer *et al.* 1981a) and a haem group which may participate in its catalytic actions, (Gerzer *et al.* 1981b; 1981c). For a full review of soluble guanylate cyclase regulation, activation and action, see Waldman & Murad (1987).

There are similarities between ANP and the above mentioned vasodilators. ANP is an endothelium independent vasodilator (Winquist *et al.* 1984a). ANP relaxes angiotensin II-induced contractions more efficiently than KCl-induced contractions (Winquist *et al.* 1984b). ANP increases levels of cGMP in various tissues, (Hamet *et al.* 1984; Hirata *et al.* 1984). However, ANP activates the particulate form of guanylate cyclase, (Tremblay *et al.* 1985a; Winquist *et al.* 1984b) and the vasodilators activate the soluble cytosolic form of guanylate cyclase, (Winquist *et al.* 1984b).

Particulate guanylate cyclase can be classed into two groups (Waldman & Murad 1987), one which is readily soluble by detergents such as Triton-X-100 and one which is detergent resistant. The former enzyme is reported to be a large glycoprotein with a molecular weight of 200-400 kDa (Limbird & Lefkowitz 1975; Goldberg & Haddox 1977) and has been highly purified from rat lung (Waldman *et al.* 1985; Kuno *et al.* 1986b) with an apparent molecular weight of 200-300 kDa. It is possible that this enzyme exists as a dimer in the cell membrane since SDS-PAGE revealed a molecule with a molecular weight of 130 kDa. The second form appears to be a different isoenzyme which is resistant to solubilisation by detergents, EDTA, salt and 1 M urea. It can be found associated with the microvillus brush border of the intestinal mucosa (Waldman *et al.* 1986) and is most likely to be associated with cytoskeletal structures within the cell (Garbers 1989). Particulate guanylate cyclase is not stimulated by nitric oxide (Tremblay *et al.* 1985b), but can be activated by the heat-stable enterotoxin of *Escherichia coli* ST (Field *et al.* 1978), (which only stimulates the detergent insoluble form of particulate guanylate cyclase). As already mentioned a detergent-soluble particulate guanylate cyclase activity has been co-purified with

ANP receptors from bovine adrenal cortex, (Takayanagi *et al.* 1987b) and solubilised plasma membranes from lung (Kuno *et al.* 1986b). This has led to the view that ANP binding and guanylate cyclase activity occupy the same transmembrane glycoprotein, (see section 1.2.5.2). ANP has also been observed to stimulate particulate guanylate cyclase activity in various tissues including, adrenal cortex (Waldman *et al.* 1984; Tremblay *et al.* 1985a; Takayanagi *et al.* 1987a), kidney (Waldman *et al.* 1984), liver (Kurose *et al.* 1987; Waldman *et al.* 1984), testes (Marala & Sharma 1988) and lung (Kuno *et al.* 1986a), as well as several cultured cell lines (Leitman & Murad 1986).

There is some interest in utilising urinary levels of cGMP as a biological marker for the action of ANP (Wong *et al.* 1988; Heim *et al.* 1988; Cogan *et al.* 1989). However it has to be remembered that levels of urinary cGMP reflect not only the rate of production by the two identified forms of guanylate cyclase but also its degradation by phosphodiesterases inside and outside the cell (Hamet *et al.* 1986). Increasing intracellular levels of cGMP, as a result of stimulation by ANP are thought to induce vaso-relaxation, (Rapoport *et al.* 1985; 1986). However there is conflicting evidence suggesting that cGMP does not mediate ANP vasorelaxant actions (Budzik *et al.* 1987). In addition there is evidence that the ANP-B receptor may not signal entirely through the production of cGMP, (Ganguly *et al.* 1989; Gupta 1989). These observations suggest that the physiological and pharmacological effects may be mediated by the ANP-C receptor population via interactions with signalling molecules such as guanosine nucleotide binding proteins (Anand-Srivastava *et al.* 1986) or the ANP-B receptor may activate other second messengers. ANP has been shown to inhibit phosphorylation of proteins (Pandey *et al.* 1987b;

Elliot and Goodfriend 1985) in adrenal cells however Ganguly *et al.* (1989) observed no difference in protein phosphorylation in adrenal glomerulosa cells stimulated by Ang II in the presence or absence of ANP.

1.2.4 Function of the ANP-C receptor

What is the function of this receptor? There have been two suggestions; 1). a new, as yet unidentified 2nd messenger is coupled to the ANP-C receptor and 2). this receptor is involved in the sequestration and clearance of ANP from the circulation, (Maack *et al.* 1987). In general, the ANP-C receptor accounts for the majority of ANP binding sites in most cells/tissues and there is no correlation with ANP binding to this receptor population and the stimulation of cGMP production, (Leitman *et al.* 1985). The predominance of ANP-C receptors over ANP-B receptors in most tissues, may explain the linear Scatchard plots often observed in [^{125}I]-ANP binding experiments. Currently, there are no available antagonists for ANP, however analogues are available which have differing affinities for ANP-B or ANP-C receptors and are very useful tools for the investigation of these receptors. The ANP-C receptor has a reduced ligand binding specificity and the carboxy-terminal phenylalanine-arginine residues of ANP are not required for binding. Studies have revealed that the most important residues for ANP binding to ANP-C receptor in cultured bovine aortic smooth muscle (Maack *et al.* 1987) and bovine endothelial aortic cells (Scarborough *et al.* 1986) are contained within a small segment of the ANP ring structure. Scarborough *et al.* (1989) prepared a series of novel ANP-C receptor specific analogues with conformational changes introduced into the peptide. In these studies, the minimal ANP amino acid sequence which retained high affinity binding to the ANP-C receptor was an 8 amino acid

segment of the 17 amino acid disulphide loop structure, (Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-NH_2). Scarborough *et al.* (1989) also suggested that that conformational adaptability of an ANP analogue, in a particular part of the sequence could be an important factor of the ANP-C receptor binding process. The ability of the ANP-C receptor to bind a variety of ligands, which now includes ANP, BNP, CNP and iso-rANP (Sudoh *et al.* 1988a; 1988b; Sudoh *et al.* 1990; Flynne *et al* 1989; Scarborough *et al.* 1989) is an unusual feature. The physiological significance of this reduced structural selectivity of ANP-C receptors remains uncertain. These investigations open up the field for the development of analogues which can avoid the process of clearing through the ANP-C receptor thus providing a means of increasing circulating levels of endogenous plasma ANP concentrations.

Recently, Anand-Srivastava *et al.* (1989) and (1990) observed that ANP inhibited adenylate cyclase activity in rat platelet membranes. Only the ANP-C receptor population is present in rat platelets (Leitman & Murad 1987). The physiological significance of platelet ANP receptors has yet to be established. Anand-Srivastava *et al.* (1990) also showed inhibition of adenylate cyclase activity in a dose-dependent manner (with ANP and des [QSGLG] ANP (4-23)-NH_2), in anterior pituitary, aorta, brain striatum and adrenal cortical membranes. This observation suggests that in these membranes ANP-C receptors may be negatively coupled to adenylate cyclase. Anand-Srivastava *et al.* (1990) speculated that ANP receptor coupling to adenylate cyclase was via an inhibitory guanine nucleotide regulatory protein (G_i). It should be borne in mind that Geiger *et al.* (1990) in rat anterior pituitary and Cramb *et al.* (1987) in rat sarcolemmal membranes reported no inhibition of adenylate cyclase by ANP.

To fully understand the nature of ANP receptor heterogeneity the use molecular biology techniques (e.g. molecular cloning and expression) are required.

1.2.5 Cloning and Expression of ANP receptors

1.2.5.1 ANP-B receptor

The isolation, sequence and expression of a cDNA clone encoding the membrane form of guanylate cyclase from the rat brain was recently reported (Chinkers *et al.* 1989). The deduced amino acid sequence of the this receptor suggests an amino-terminal signal sequence and a single transmembrane domain that divides the protein into a 441 amino acid N-terminal extracellular domain and a 567 amino acid C-terminal intracellular domain. The receptor has an apparent molecular weight of 115,852Da and has six cysteine residues and six potential N-glycosylation sites in the extracellular domain. Regions of the intracellular domain are related to the catalytic domain of protein kinases, and also share 42% homology with the amino acids of the carboxy-terminal subunit of the bovine soluble form of guanylate cyclase, Chinkers *et al.* (1989). Expression of this cDNA clone in COS-7 cells transfected using the mammalian vector pSVL, produces a membrane protein that binds ANP with high affinity and also has endogenous guanylate cyclase activity. Lowe *et al.* (1989) isolated cDNA's encoding the human ANP-B receptor from placenta and kidney and expressed the cDNA clones in COS-7 cells transfected with the vector pRK (this group termed the ANP-B receptor as the ANP-A receptor). This ANP-B receptor showed 90% amino acid sequence homology to the above mentioned rat brain ANP-B receptor. Pandey & Singh (1990) have isolated a murine Leydig cell ANP-B receptor which shows 97% amino acid sequence homology to the rat brain ANP-B

receptor and 94% amino acid sequence homology to the ANP-B receptor isolated from human placenta (Lowe *et al.* 1989). Chang *et al.* (1989) reported expression, in COS-7 cells transfected with the vector pRK of a second placental human natriuretic peptide/guanylate cyclase receptor. This second ANP-B receptor was shown at this time to be preferentially activated by BNP, (a 9.7 fold increase in cGMP) rather than ANP, (a 3 fold increase in cGMP). They also reported that the ANP-B receptor of Lowe *et al.* (1989), in terms of binding and guanylate cyclase activation, responded similarly to both ANP and BNP (2 and 1.5 fold increases in cGMP respectively). These two receptors will generally be referred to as ANP-B1 receptor (previously referred to as human ANP-A receptor by Lowe *et al.* 1989) and ANP-B2 receptor (previously referred to as the second human ANP-B receptor by Chang *et al.* 1989). Chang *et al.* (1989) reported this second receptor, ANP-B2 receptor to have an apparent molecular weight of 114,952 Da, with six cysteine residues and seven potential N-glycosylation sites in the extracellular domain. The receptor was also found to have a cytoplasmic domain of 569 amino acids. The extracellular domain of human ANP-B2 receptor, (Chang *et al.* 1989) showed 44% amino acid sequence homology to human ANP-B1 receptor, (Lowe *et al.* 1989). The intracellular domains of human ANP-B2 and ANP-B1 receptors showed an overall 74% homology with 63% in the protein kinase like domain and 88% in the soluble guanylate cyclase subunit homologous region. Schulz *et al.* (1989) reported the expression of two rat guanylate cyclase receptors, (termed GC-A and GC-B the rat equivalent of human ANP-B2 and ANP-B1 receptors respectively) in COS-7 cells transfected with the vector pSVL (these two cyclases showed an overall 62% amino acid sequence homology). The intracellular domains of these two receptors showed 78% amino acid sequence

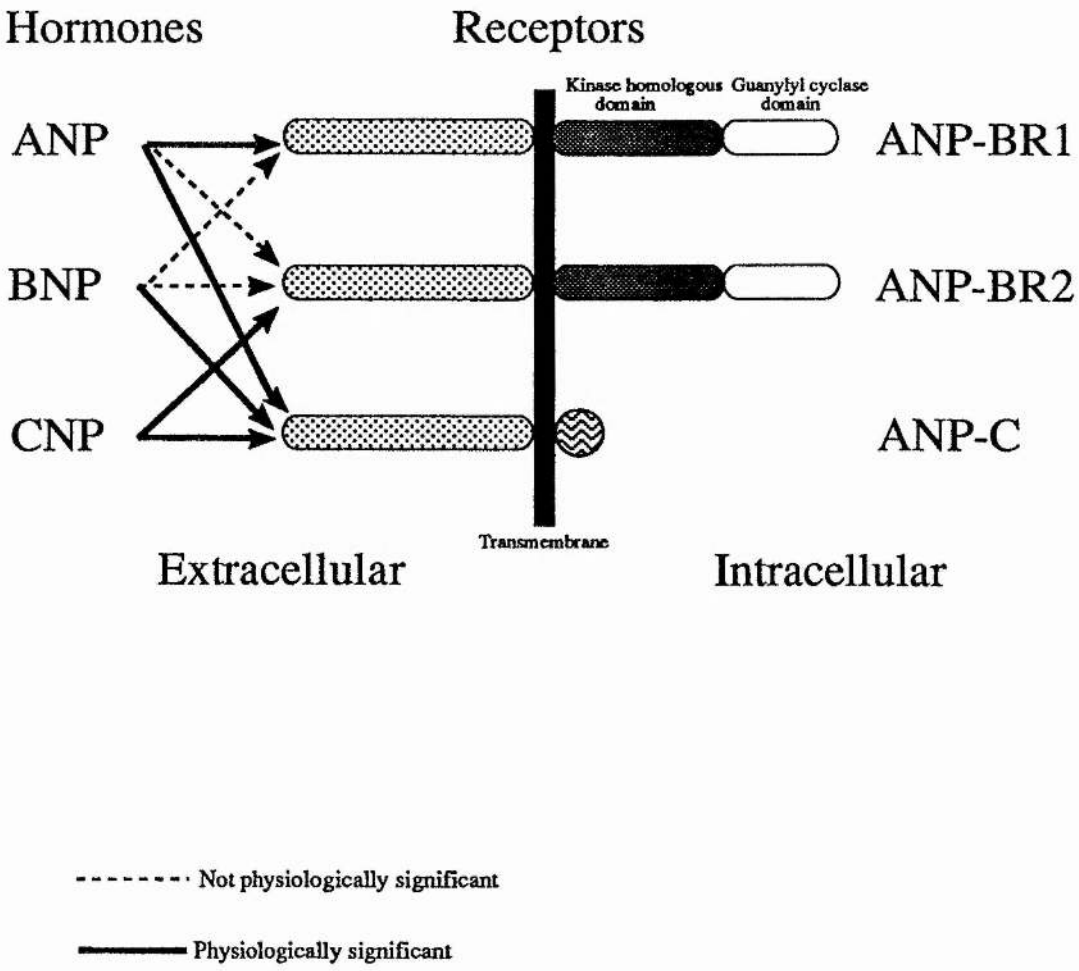
homology (to each other) and the extracellular domains a 43% amino acid sequence homology. More recently, results from Koller *et al.* (1990) indicated that the newly discovered CNP was the most likely physiological ligand for the ANP-B2 receptor and that relatively high concentrations (500nM) of BNP were previously required to elicite a cGMP response with this receptor, Schulz *et al.* (1989) and Chang *et al.* (1989) (see fig. 1.4).

Recently, Schulz *et al.* (1991) identified a unique form of guanylate cyclase enzyme that does not recognise natriuretic peptides. This enzyme clone, termed GC-C was isolated from an intestinal cDNA library and expressed in COS-7 cells. GC-C was shown to exhibit the general structural features of ANP-B1 and ANP-B2, however showed little amino acid homology compared to the extracellular domains of ANP-B1 and ANP-B2. In addition GC-C was shown not to be stimulated by ANP, BNP or CNP but was stimulated by the heat-stable enterotoxin of *Escherichia coli* ST and specifically bound ^{125}I-labelled *Escherichia coli* ST. These results indicate the presence of at least one form of particulate guanylate cyclase which is not associated with natriuretic peptide binding.

To summarise, the plasma membrane forms of ANP-B receptors contain at least three distinct domains; extracellular ligand binding, intracellular catalytic protein-kinase like domain (similar to that seen in growth factor receptors such as platelet derived growth factor (PDGF)). Chinkers *et al.* (1989) showed that a 256 amino acid portion of the intracellular domain of their ANP-B1 receptor was 31% identical to the protein kinase domain of PGDF receptor. Finally an intracellular domain which is homologous to a subunit of a bovine soluble form of guanylate cyclase. ANP-B1 and

Figure 1.4

Hormone specificity for the human natriuretic peptide receptors, ANP-BR1, ANP-BR2 and ANP-C.



ANP-B2 receptors are a single transmembrane class of receptors which function uniquely by coupling to endogenous guanylate cyclase activity and may exist as a family of many members all varying in their ligand binding domains.

1.2.5.2 ANP-C receptor

In 1988, Fuller *et al.* (1988) isolated, sequenced and expressed cDNA clones encoding the bovine ANP-C receptor. The full length amino acid sequence of the ANP-C receptor was established from these clones and was found to be synthesised as a 537 amino acid precursor with an apparent molecular weight of 59,744 Da. Amino acid analysis of the cDNA suggested that the mature form of this receptor consists of a 496 amino acid extracellular domain consisting of the ANP binding site, a single hydrophobic transmembrane anchor of 23 amino acids and a short carboxy-terminal cytoplasmic tail of 37 amino acids. A cDNA clone was transcribed *in vitro* and the resulting synthetic mRNA microinjected into *Xenopus* oocytes. These in turn expressed an endogenous ANP receptor, possessing high affinity binding for ANP, truncated $ANP_{(103-123)}$ and des [QSGLG] ANP (4-23)-NH_2. These observations were supported by Porter *et al.* (1988; 1989). Porter *et al.* (1989) utilised a vaccinia virus expression system transfected with the plasmid pSC11 to characterise a mutant receptor which lacked the transmembrane and cytoplasmic domains of the ANP-C receptor. Their results indicated that the extracellular domain of the ANP-C receptor is sufficient for high affinity binding since the mutant receptor had binding affinities for ANP and des [QSGLG] ANP (4-23)-NH_2 identical to that of the native ANP-C receptor. The cDNA sequence of the human ANP-C receptor has also been elucidated, (Lowe *et al.* 1990; Porter *et al.* 1990) and is found to comprise of a 540/541 amino

acid precursor with an apparent molecular weight of 59811Da. This human ANP-C receptor has 93/95% amino acid sequence homology with the bovine ANP-C receptor, Fuller *et al.* (1988) and 33/35% amino acid sequence homology with the rat and human ANP-B1 receptor, Chinkers *et al.* (1989) and Lowe *et al.* (1989) respectively.

The above information therefore indicates the presence of a unique family of related natriuretic/diuretic peptides in co-existence with an equally unique family of natriuretic/diuretic peptide ligand binding receptor sites. It therefore remains to summarise the mechanisms controlling the release of ANP and its subsequent physiological actions and finally, to examine the role of ANP in hypertension.

1.3 ANP RELEASE AND PHYSIOLOGICAL ACTIONS

1.3.1 Mechanisms controlling the release of ANP

In general the release of hormones from cells is thought to be regulated by three internal pathways, (Lang *et al.* 1987). One employs ATP and the enzyme adenylate cyclase, then generating the second messenger cAMP. A second employs calcium ions, where cytosolic calcium can be increased by calcium entering the cell through selective membrane channels or be mobilised from intracellular stores, e.g. the endoplasmic reticulum. Calcium release from these intracellular stores is initiated by inositol 1,4,5-triphosphate (IP_3), which is generated from membrane phosphoinositides along with diacylglycerol (DAG). IP_3 and DAG form part of the third internal pathway, the phosphatidylinositol (PI) system. Protein phosphorylation, mediated by protein kinases, is one of the final steps of all the pathways and may facilitate the transport and fusion of secretory granules with the cell membrane. Cyclic AMP (cAMP) and DAG

are responsible for the activation of protein kinase A and C respectively. The activation of protein kinase C requires calcium which is mobilised from intracellular stores by IP_3. Thus, IP_3 and DAG are synergistic in the activation of protein kinase C. The use of pharmacological agents which mimic the actions of specific second messengers can be used to assess the part played by each pathway in mediating hormone release. Lang *et al.* (1987) investigated the effects of activation of the calcium calmodulin pathway by A23187 (a calcium ionophore) and BAYK8644, (a calcium channel agonist) on the release of ANP. Both A23187 and BAYK8644 introduce free calcium into the cell therefore they can be used to mimic the actions of IP_3. Lang *et al.* (1987) also investigated the effects of 12-*O*-tetradecanoylphorbol-13-acetate (TPA, a phorbol ester which stimulates protein kinase C) and forskolin (which is known to activate adenylate cyclase and cause an increase in cAMP levels) on the release of ANP. They found that all of the above mentioned pharmacological agents had stimulatory effects on ANP secretion and that TPA plus A23187 or BAYK8644 had a more than additive effect. These results suggested that ANP secretion is a calcium dependent process and that elevated calcium may contribute to the activation of protein kinase C (Lang *et al.* 1987).

The release of ANP from the heart is therefore stimulated by cytosolic calcium concentrations, which in turn can be augmented by cAMP and protein kinase C (Lang *et al.* 1987). The concentration of calcium in the heart is dependent upon many factors, including the action of various humoral substances, cardiac nerve activity, heart rate and the mean length of heart fibres. This invites speculation that these factors may also play a role in the regulation of ANP release.

Indeed there is evidence that ANP release is increased by adrenalin and arginine vasopressin, (Sonnenberg *et al.* 1984; Sonnenberg & Veress 1984).

A role for the autonomic nervous system in the regulation of ANP release has also been suggested since it has been observed that acetylcholine, (Sonnenberg & Veress 1984) and catecholamines, (Lang *et al.* 1987) both stimulate the release of ANP from rat atria *in vitro*.

Increased plasma ANP levels are found during tachycardia or when atrial pacing is maintained at high frequencies (Tikkanen *et al.* 1985). The release may be directly due to the increased heart rate or may possibly result from an increase in atrial filling pressure increasing atrial tension. ANP plasma concentrations have been reported to be elevated in patients with congestive heart failure (Tikkanen *et al.* 1985), and children with congenital heart diseases and bronchopulmonary abnormalities (Lang *et al.* 1985). These conditions are associated with increased atrial filling pressure which causes distension of the atrial wall. Dietz (1984) has shown a positive correlation between ANP secretion rate and atrial filling pressure in man (patients with coronary heart disease) and rats (animals subjected to acute volume loading). Fluid (Lang *et al.* 1985) and salt loading (Sagnella *et al.* 1985) have also been shown to increase right atrial pressure with a rise in ANP plasma concentration. Changes in posture, which affects venous return (Hodsman *et al.* 1985) and head-out water immersion (Katsube *et al.* 1985), have also been shown to be associated with increases in plasma ANP concentration and are correlated with an elevation in right atrial pressure. *In vivo* atrial stretch has also been observed to induce ANP release, (Ledsome *et al.* 1985).

Humoral, cardiac nerve activity and heart rate factors are all indicated in ANP release however, myocardial fibre length is possibly the most important factor. Secretion of ANP may be influenced by the rise in free intracellular calcium which is observed when myocardial fibre length is increased, (Lang *et al.* 1987).

1.3.2 Physiological effects of ANP

The observation by De Bold *et al.* (1981), of a vast increase in renal sodium and water excretion initiated by an atrial protein, (ANP) led to much experimentation on the mechanisms by which ANP mediates this response. ANP has been shown to have a number of actions when studied *in vitro* and *in vivo*. These actions include an inhibition of renin and aldosterone secretion, relaxation of preconstricted smooth muscle and alterations in renal vascular haemodynamics. (The latter, although an important effect of the physiological action of ANP will not be discussed in this thesis due to the vast amount of information available and the difficulty involved in condensing this information to a brief summary).

1.3.2.1 The Effects of ANP on Renin Release

When ANP is secreted from cardiocytes in response to a release stimuli such as atrial stretch, it is transported via the arteries, to its various target tissues. Once ANP has reached these targets its primary role is to modify the mechanisms which regulate blood pressure and blood volume. In particular ANP interacts with the renin-angiotensin system. Renin is secreted by juxtaglomerular cells into the bloodstream when blood pressure and sodium concentration in the kidney tubule is low (Cantin & Genest 1986). Renin cleaves the plasma protein angiotensinogen which

in turn forms the decapeptide angiotensin I which is converted to the octapeptide angiotensin II by angiotensin converting enzyme (ACE) which is mainly found in the lung. Angiotensin II is a powerful vasoconstrictor which suppresses further release of renin from juxtaglomerular cells and stimulates the release of aldosterone from the adrenal gland. ANP affects the renin-angiotensin system by inhibiting the release of renin and by directly inhibiting the adrenal secretion of aldosterone. The exact mechanisms by which ANP suppresses renin release are not as yet fully understood and are controversial. Opgenorth *et al.* (1986) have shown that ANP does not suppress renin secretion in the non-filtering kidney, this suggested that the suppressive mechanism may involve the macula densa cells of the distal tubule. Recently, Kagayama and Brown (1990) have shown that ANP renin suppression is a calcium independent mechanism.

Although the general consensus is that ANP suppresses the release of renin (Kagayama & Brown 1990; Cody *et al.* 1986; Kurtz *et al.* 1986), renin has also been reported to be increased (Hiruma *et al.* 1986) or remain unchanged (Rodeiguez-Puyol *et al.* 1986) after administration ANP.

1.3.2.2 The Effects of ANP on Aldosterone Release

In contrast to the conflicting evidence concerning ANP's actions on renin secretion, Atarashi *et al.* (1984), (using crude atrial homogenates) and Chartier *et al.* (1984), DeLean *et al.* (1984) and Goodfriend *et al.* (1984), (using synthetic peptides) have shown that ANP directly inhibits the secretion of aldosterone from the adrenal gland. In the same year Chartier *et al.* (1984) observed in rat zona glomerulosa cells, that angiotensin II (AngII), adrenocorticotrophic hormone (ACTH) and

potassium chloride (KCl) stimulated aldosterone secretion was inhibited by ANP and in primary cultures of bovine zona glomerulosa cells ANP showed up to 70% inhibition of AngII, ACTH, prostaglandin and forskolin stimulated aldosterone secretion. Atarashi *et al.* (1984) showed evidence of a preferential inhibition of ANP on AngII-stimulated secretion, since a high concentration of AngII (10^{-8}M) was unable to overcome the inhibitory effect of ANP on aldosterone release. There is evidence (Goodfriend *et al.* 1984; Elliot & Goodfriend 1986; Kudo & Baird 1984) to suggest that the mechanism of ANP inhibition is at some early part of the steroidogenic pathway, before mitochondrial uptake and metabolism of cholesterol. Lawrence *et al.* (1990) has shown in humans on a low salt diet and under induced hypoxic conditions, increases in plasma ANP which coincide with decreases in plasma aldosterone. How this depressive action on aldosterone secretion contributes to the actions of ANP remains to be established. High and low affinity receptors for ANP, which are distinct from ACTH and AngII receptors, have been identified in bovine adrenal zona glomerulosa membranes (DeLean *et al* 1984, Meloche *et al.* 1987a; 1987b) and it is likely that these mediate the effects of ANP on the cells.

1.3.2.3 Vascular effects of ANP

Deth *et al.* (1982) and Currie *et al.* (1983) have suggested that the hypotensive actions of ANP may be mediated via vasorelaxation. These two groups reported the relaxation of aortic strips *in vitro* by rat atrial extracts. Subsequently, it was confirmed that extracted ANP (Garcia *et al.* 1984) and synthetic ANP (Atlas *et al.* 1984; Garcia *et al.* 1984; Cohen & Schenk 1985) act in a similar manner. The general vasorelaxant response of ANP is independent of the vasoconstrictor and similar results are

found with AngII, noradrenaline, histamine and caffeine. There have however, been variable results observed with the actions of ANP on KCl-induced vasoconstriction (Garcia *et al.* 1984; Rapoport *et al.* 1985 & Bratveit *et al.* 1987). The actions of ANP on KCl-induced vasoconstriction are not yet fully understood, however it is thought that reported differences may be due to the various alterations in intracellular calcium (Ca^{2+}). AngII and noradrenaline act by releasing Ca^{2+} from intracellular stores e.g. the endoplasmic reticulum (Deth & Van Breeman 1977; Van Breeman *et al* 1982). Chui *et al.* (1986) and Meisheri *et al.* (1986) showed that ANP inhibited agonist stimulated intracellular Ca^{2+} release and Ca^{2+} influx, however they found that ANP had no effect on KCl-stimulated Ca^{2+} influx. This is perhaps because the membrane depolarisation induced by high potassium is not as sensitive to the relaxant effect of ANP compared to that of AngII and noradrenaline induced contractions (Chui *et al.* 1986).

The vasorelaxant actions of ANP are generally considered to be mediated by increases in intracellular cGMP (Rapoport *et al.* 1985; 1986). The increases in cGMP causes activation of a cGMP-dependent protein kinase which may lead to the inhibition of Ca^{2+} translocation through agonist or receptor-operated Ca^{2+} channels and/or enhance Ca^{2+} extrusion via activation of the sarcolemma extrusion pump (Popescu *et al.* 1985) and/or interfere with the release of Ca^{2+} from intracellular storage sites. Ultimately, the result is a decrease in Ca^{2+} and vasorelaxation. However recently, Budzik *et al.* (1987) studying the effects of various ANP analogues in cultured vascular smooth muscle cells observed dissociation between ANP analogue stimulated increases in cGMP and ANP analogue vasorelaxation of preconstricted rabbit aorta. The extent of vasorelaxation

is dependent upon the vascular preparation (Garcia *et al.* 1984; Cohen & Schenk 1985) and the analogue of ANP used. The ring structure of ANP is known to be important for binding, (Misono *et al.* 1984a; 1984b), and changes at the C-termini of ANP have been shown to decrease the vasorelaxant effect of ANP, (Garcia *et al.* 1985c). In general, the vasorelaxant activities of ANP are more effective in arterial rather than venous preparations and this correlates with numbers of receptors in these tissues (Cohen & Schenk 1985). Winquist *et al.* (1985) found large numbers of high-affinity ANP B-receptors in both aorta and renal artery, intermediate numbers in pulmonary artery and vein and low numbers in ear and femoral arteries which all correlated with the dose dependent vasorelaxation response to ANP. However this group also found large numbers of high-affinity ANP B-receptors on renal and jugular veins, with a poor correlation between ANP receptor binding and relaxation response. Therefore differences in receptor localisation and/or density cannot solely account for the observed differences in vasorelaxation of isolated vascular preparations.

In vivo animal studies are not in complete agreement with *in vitro* studies. Some reports indicate that administration of ANP results in a decrease (Volpe *et al.* 1986), an increase (Koike *et al.* 1984), or no change (Lappe *et al.* 1985) in total peripheral resistance. However administration of ANP (atrial extract or analogues of ANP) has generally been shown to cause a reduction in mean arterial pressure (Pegram *et al.* 1986), with the greatest reductions noted in studies on hypertensive animals. In the human, Richards *et al.* (1985) observed symptoms of arterial vasodilation (flushing, reflex tachycardia, increased skin blood flow) after injection of ANP in healthy volunteers.

1.4 ATRIAL NATRIURETIC PEPTIDE AND HYPERTENSION

1.4.1 Hypertension

Hypertension (high blood pressure) is defined as a chronically increased arterial pressure. Abnormally high blood pressure (BP) may be associated with several known diseases (Secondary Hypertension), or may occur spontaneously with no apparent clinical disorder being diagnosed (Essential or Primary Hypertension). In the case of secondary hypertension the increase in blood pressure is a result of some disorder/cause which can be clearly identified, e.g. drug induced, neurogenic and renal disorders or can be associated with hormonal changes induced by pregnancy. In the case of essential hypertension which accounts for 80-95% of all patients with hypertension, the actual cause is unknown. It is this type of hypertension which has been studied most closely with regard to ANP and its natural hypotensive actions in resisting increases in intravascular volume and blood pressure. A widely used scheme grading levels of essential hypertension (Bowman & Rand 1980) is as follows; *Grade 1 mild hypertension*; patient BP is considered to be above the normal values of 120/80mmHg (systolic/diastolic) but is consistently less than 170/110mmHg; *Grade 2 moderate hypertension*, patient BP is greater than 170/110mmHg and there evidence of left ventricular hypertrophy; *Grade 3 severe hypertension*, patient BP is greater than 170/110mmHg and there are signs of marked left ventricular hypertrophy and of impaired renal function, in particular a serum creatinine concentration exceeding 15mg/l and; *Grade 4 malignant hypertension*, patient show a high and rapidly increasing BP, diastolic pressure at this time is usually greater than 130mmHg and the condition is associated with retinal haemorrhages and occasionally complications

such as heart failure, stroke and renal impairment. This grade of hypertension is usually fatal to 90-95% of patients if untreated.

Sugawara *et al.* (1985) and Sagnella *et al.* (1986) have shown that in patients with a *moderate degree* of essential hypertension there is an associated increase in plasma ANP concentration and there appears to be a correlation between atrial pressure and plasma ANP concentration as the levels of hypertension progress. Sagnella *et al.* (1986) and Richards *et al.* (1986) have also reported increases in plasma ANP concentration with age in normotensives and BP is known to rise with age (Beevers 1987). However there remains a great deal of controversy in reports concerning *mild* essential hypertension. Yamaji *et al.* (1986), Larochelle *et al.* (1987) and Nilsson *et al.* (1987) all agreed that there was no change in plasma ANP concentration in patients with *mild* untreated essential hypertension, compared to normotensive control subjects. Conversely, Montorsi *et al.* (1987) showed a significant increase in ANP levels (45 ± 3 pg/ml compared to 36 ±3 pg/ml) with a *mild* hypertensive group of patients. An important factor to consider when analysing these results is the degree of standardisation between the groups since plasma ANP concentration can vary with all of the following factors; dietary sodium intake (Tanaka *et al.* 1984); age (Beevers 1987); posture (Hodsman *et al.* 1985) and possibly race and physical ability. There is also some speculation that during the early stages of development of certain types of essential hypertension there may be a deficiency in secretion of ANP with a concomitant increase in blood pressure which may play a primary role in inducing, maintaining or enhancing the effects of hypertension. This hypothesis is supported by Ferrier *et al.* (1988), who examined the children of hypertensive patients with regards to their response to an

increase in dietary sodium. Results of this study indicated a lack of sodium induced increase in plasma ANP concentration compared to children of normotensive subjects. This report was supported by Tunny *et al.* (1986) who examined Gordon's syndrome (hypertension is present associated with hyperkalaemia and volume expansion), where plasma ANP concentration were near to normal perhaps indicating an attenuation of response of plasma ANP. Schiffrin *et al.* (1988) has studied in platelets isolated from patients with essential hypertension, the relationship between plasma ANP concentration and ANP receptor density (platelets are known to express only the ANP-C receptor population). This group reported a decrease in the density of ANP receptors in hypertensive patients with an inverse correlation to plasma ANP concentration. Administration of ANP in varying pharmacological doses to patients suffering from essential hypertension has revealed various results, (for a fuller review see Richards (1990). It is generally accepted that prolonged administration of physiological doses of ANP (0.5-2 pmol/kg/min) results in a fall in arterial blood pressure with a mild negative alteration in sodium balance, without activation of the reflex counterbalancing mechanisms, such as stimulation of the renin-angiotensin-aldosterone system (RAAS) and sympathetic nervous system. In addition there are no signs of the extensive hypotension/bradycardia noted with high ANP infusion doses such as 64-144 pmol/kg/min (Weder *et al.* 1987). These observations open the way for the use of ANP, ANP-analogues or agents which prevent the degradation of endogenous ANP in the treatment of hypertension.

To examine the relationship between ANP and hypertension various animal models have been studied as well as studies in the human.

Changes in the density and/or the affinity of target organ/tissue receptor sites for ANP and plasma ANP concentration may alter as hypertension develops.

1.4.2 The effects of salt-loading and dehydration in Sprague-Dawley rats

Marie *et al.* (1976) and De Bold (1979) demonstrated that water loading and increasing sodium caused significant decreases in the number of atrial ANP containing granules. This initial observation has led to the investigation of the effects of salt and water dietary alterations on plasma ANP concentration, ANP receptor density, tissue and plasma cGMP levels and BP in many animal models.

Kollenda *et al.* (1990) investigated the short term (3 days) effects of salt loading (1% saline in drinking water) in male Sprague-Dawley rats. They reported that salt loading did not induce any significant change in plasma ANP concentration or in ANP receptor density in glomeruli membranes. This result was supported by Morton *et al.* (1987) who studied alterations in sodium diet (over 10 days) in male Sprague-Dawley rats. They showed no change in plasma ANP concentration with high (1% saline in drinking water) or low (0.002% saline in drinking water) salt diets in male Sprague-Dawley rats. Ballerman *et al.* (1985) examined the effects of both salt depletion and administration of isotonic saline after 14 days, on glomerular ANP receptor density in male Sprague-Dawley rats. They concluded that a high salt diet resulted in a reduction (4-fold) in glomerular ANP receptor density and an increase in receptor affinity relative to the values for the low salt diet rats. There was no change in glomerular ANP-induced cGMP production with the different diets. Plasma ANP concentration was 132 ± 63 pM and 23 ± 5 pM in the high salt

and salt depleted rats respectively. Ballerman *et al.* (1985) speculated that the altered glomerular ANP receptor is not directly linked to guanylate cyclase and that alterations in body fluid volume may be reflected in a decrease in glomerular ANP-C receptor density. Michel *et al.* (1990) investigated salt loaded (0.9% saline in drinking water) male Sprague-Dawley rats after 35 days. Plasma ANP, renal function, BP and cGMP levels all remained the same, compared to control rats receiving deionised water, however this group did observe a down-regulation of receptor numbers (predominately ANP-C receptors) in isolated renal glomeruli compared to control rats. Widimsky *et al.* (1990) also examined the long term effects of salt loading in male Sprague-Dawley rats. These investigators reported that a prolonged high-salt diet (8% w/w NaCl rat chow) after 5 wk but not 3 wk, caused an increase in metabolic clearance rate and volume distribution of [^{125}I-ANP] when compared to control rats, (0.8% w/w NaCl rat chow). They also noted a greater [^{125}I]-ANP uptake in various tissues after 5 wk but not after 3 wk, on the high salt diet. From their results they hypothesised that prolonged salt ingestion increases the density and/or affinity of ANP binding sites, (most probably ANP-C receptors) and that this may explain the previously observed (Debinski *et al* 1988) decreases in plasma ANP concentration after prolonged salt ingestion (5wk).

The general consensus (with the exception of Widimsky *et al.* 1990) is that an increased salt diet causes a decrease in glomeruli ANP receptor density in the Sprague-Dawley rat. The indications are that time is an important factor in causing, most likely ANP-C receptor down-regulation.

Schwartz *et al.* (1986) showed that three days of water deprivation caused a significant decrease in plasma ANP concentration compared to controls. This observation was supported by Kollenda *et al.* (1990). They observed that dehydration, (no water for 3 days) in male Sprague-Dawley rat glomeruli, leads to a decrease in plasma ANP concentration and an increase in ANP-C receptor density. They speculated that the inverse relationship between plasma ANP concentration and ANP-C receptor density could be an adaptive mechanism for maintaining fluid homeostasis.

1.4.3 Genetic experimental models for Hypertension

1.4.3.1 The Spontaneously hypertensive rat (SHR)

Sonnenberg *et al.* (1983) reported that the ANP concentration per atria of SHR was lower than in normotensive Wistar-Kyoto (WKY) control rats. Radioimmunoassay (RIA) showed that the ANP concentration per unit weight of atrium was similar in both strains at weaning but as hypertension developed with age, the ANP concentration in the left atria of SHR decreased relative to WKY with the ANP concentration in the right atria remaining constant. Higa *et al.* (1985) and Takayanagi *et al.* (1986) also showed similar results with age developed hypertension in SHR. The plasma ANP concentration in the study of Higa *et al.* (1985) was reported to be lower in SHR than in WKY. However in contrast, Imada *et al.* (1985), Takayanagi *et al.* (1986) and Morii *et al.* (1986) found similar plasma ANP concentration in both strains when young but higher plasma [ANP] in SHR as the rats aged and became hypertensive.

Takayanagi *et al.* (1986) also showed that there was a decrease in ANP receptor density (B_{max}) in aortic smooth muscle membranes and adrenal

membranes in SHR, with an inconsistent increase in cGMP response even before the onset of hypertension. Conversely, in cultured aortic vascular smooth muscle cells, Nakamura *et al.* (1988) found an increase in receptor density and an increase in K_d in the SHR. However, despite the increase in receptor density for ANP there was a lower maximal stimulation of cGMP in response to ANP. The difference in receptor density of these two reports is perhaps a result of the experimental conditions used. In the report of Takayanagi *et al* (1986) the increased ANP concentration is likely to down regulate receptor density. However in the report of Nakamura *et al* (1988) there was no prior exposure to increased circulatory plasma ANP, hence no down regulation of receptor density. These result suggest that *in vivo* and *in vitro* results should perhaps be interpreted with some caution.

Saavedra *et al.* (1986b) investigated the ANP receptor population in subfornical organ (SFO), choroid plexus (CP) and the olfactory bulb (OB) in SHR and age matched WKY normotensive control rats. They reported a decrease in the number of binding sites for ANP and a lower K_d (higher affinity) for 4 wk and 14 wk SHR in the SFO, also a decrease in ANP receptor density with no difference in K_d in the CP. They found no difference in ANP receptor density or K_d with OB. These investigations were supported by Brown & Czarnecki (1990) who also reported a reduction in the number of binding sites for ANP in SHR in the SFO and in the CP but no reduction in arachnoid mater (AM). Brown & Czarnecki (1990) also showed a lower K_d in SFO and CP but no change in AM. Okazaki *et al.* (1990) examined ANP binding sites in the cerebral microvessels and in the CP of SHR and WKY. In the microvessels they found no difference in affinity but a lower B_{max} in SHR compared to

WKY and in the CP a higher affinity and a lower B_{max} in SHR compared to WKY. Garcia *et al.* (1981) and Johanson (1976) showed increases in the diameter, evidence of endothelial cell degeneration and collagen deposition in cerebral capillaries with experimental hypertension in humans and monkeys. Hypertension therefore causes changes in cerebral microvasculature and ANP receptors in cerebral microvessels and the choroid plexus may participate in the regulation of cerebral microcirculation. The SFO is a structure which lies outside the blood-brain barrier and is therefore exposed to circulating blood peptides, the SFO may then also act to regulate cerebral microcirculation. In 1987, Chabrier *et al.* (1987) speculated that ANP receptor sites in normotensive bovine brain microvessels were indicative of a potential physiological effect of ANP on brain microcirculation and/or the blood-brain barrier.

Ogura *et al.* (1987) examined renal (whole kidney) receptors of ANP in 5 wk and 12 wk SHR. They reported increases in BP, decreases in B_{max} and decreases in K_d for both groups of SHR. However, the increase in BP in the 12 wk group and the decrease in binding capacity were both greater. Garcia *et al.* (1989) investigated glomerular ANP receptors in 4, 6, 12 and 16 wk SHR. ANP receptor density was reported to be similar in 4 wk SHR and age matched WKY controls. However, ANP receptor density in SHR was reported to be lower than age matched WKY controls at 6, 12 and 16 wk. A lower K_d compared to controls was also noted at 6 and 12 wk. ANP stimulated cGMP production was reported to be lower in SHR than in WKY, suggesting a possible down-regulation in the ANP-B receptor population. Brown *et al.* (1990) using the synthetic ANP analogues, des [QSGLG] ANP (4-23)-NH_2 (des-ANP) which is specific for ANP-C receptors and rat Atriopeptin 1 (ANP(5-25)) which selectively relaxes intestinal

smooth muscle but not vascular smooth muscle stripes (Currie *et al.* 1984c), investigated ANP receptor subtypes in renal, (glomeruli, stripes in outer medulla and in inner medulla) of SHR and WKY. They showed that des-ANP and ANP(5-25) inhibited [^{125}I]-ANP binding by approximately 70% in the glomeruli but showed no inhibition of [^{125}I]-ANP binding in the medulla of both SHR and WKY. However, the receptor sites of the glomeruli and of the medulla able to bind [^{125}I]-ANP in the presence of 10μM des [QSGLG] ANP (4-23)-NH_2 were different in the SHR compared to the WKY. In the WKY ANP(5-25) was unable to displace [^{125}I]-ANP glomerular binding and did not displace more than 40% of [^{125}I]-ANP medullary binding. In contrast, in the SHR [^{125}I]-ANP binding was completely displaced by ANP(5-25). SHR glomeruli and medulla also showed a lower K_d and a lower B_{max} compared to WKY. These results suggested that ANP-C receptors are restricted to the glomeruli and not the medulla. Also that binding sites of the medulla are most likely ANP-B receptors, (guanylate cyclase coupled) and that the ANP-B receptors in SHR have, like WKY, a low affinity for des-ANP, but differ from WKY in possessing a higher affinity for ANP(5-25). The actions of ANP(5-25) in the glomeruli and the medulla of SHR indicate that ANP(5-25) although having a greater affinity for the ANP-C receptor than for the ANP-B receptor is competitively binding at another receptor site. This other receptor site maybe the ANP-B2 receptor (See 1.2.5.1) or a defect in receptor guanylate cyclase activity may somehow alter receptor binding characteristics (Brown *et al.* 1990). (These results do not rule out the presence of low numbers of B receptors in the glomeruli).

Kurihara *et al.* (1987) reported a decrease in ANP receptors in the thymus and the spleen of young (4 wk) SHR compared to age matched WKY.

ANP-stimulated cGMP production was reported to be similar, suggesting a possible down-regulation in the ANP-C receptor population in the thymus and the spleen. This group indicated that reduced ANP binding was present before the development of hypertension and that it persisted after hypertension had developed.

The above results are conflicting. There is general agreement that in the SHR there is a down-regulation of ANP receptors with a decrease in K_d, however there is controversy as to which receptor population is down-regulated and what happens to cytosolic cGMP concentration. Alterations in ANP receptor density is also implicated in organs of the immune system during hypertension. As to changes in plasma ANP levels, there is again much controversy with no real consensus to the reported results. However, it would seem that in all cases rat age is an important determinant of ANP concentration in the blood.

1.4.3.2 The Dahl hypertensive rat

Dahl *et al.* (1962) selectively bred rats for sensitivity (S) or resistance (R) to the hypertensive effect of a high salt diet, (a model of essential hypertension). The role of ANP in the development of hypertension has been examined in these rats, although not as extensively as in the SHR. In contrast to the SHR rat and WKY control rats, Dahl-S rats have been shown to have higher ANP concentration in their atria (Snajdar & Rapp 1985), than Dahl-R rats. Snajdar & Rapp (1985), reported that in young rats (1-2 month), the ANP content of the heart was higher in Dahl-S than Dahl-R rats with small differences in BP, (113 ± 2.9 mmHg and 103 ± 2.1 mmHg respectively). In addition, in older rats (7 month) where the BP differences were greater between Dahl-S and Dahl-R rats, (234 ± 7.1 mmHg

and 130 ± 2.3 mmHg respectively), ANP in the atria still remained higher in Dahl-S rats than in Dahl-R rats. Alterations of diet from normal, 1% salt-chow to low, 0.3% or high, 8% salt-chow did not affect this difference. An 8% salt diet equally suppressed the ANP atrial content of both strains. Snajdar and Rapp (1985) also reported renal hypo-responsiveness to ANP in 1 month pre-hypertensive Dahl-S rats compared to age-matched Dahl-R rats. In contrast however as the rats aged (7 month) and became markedly hypertensive Dahl-S rats showed hyper-responsiveness to ANP compared to age-matched Dahl-R rats. In 1986, Schwartz *et al.* (1986) and Gutkwoska *et al.* (1986) investigated the effects of salt diet on BP and plasma ANP concentration in Dahl rats. Both of these groups reported that Dahl-S rats fed on a high salt diet, (5 weeks on 8% salt) have a higher blood pressure and higher plasma ANP concentration than Dahl-R rats, on the same diet. Snajdar & Rapp (1986), further examined the effects of age and diet on Dahl-S and Dahl-R rats. In this study Dahl-S and Dahl-R rats (2 month of age) on a normal salt diet again showed small differences in BP (119 ± 3.6 mmHg and 111 ± 2.6 mmHg respectively) with no statistical differences in plasma ANP concentration (222 ± 22.2 pg/ml and 251 ± 20.3 pg/ml respectively). In contrast to this, 6 month old Dahl-S rats showed increased BP when compared to Dahl-R rats, 199 ± 5.3 mmHg and 119 ± 2 mmHg respectively and increased plasma ANP concentration, 1079 ± 259.3 pg/ml and 242 ± 34.4 pg/ml respectively. Snajdar & Rapp (1986) also showed that changes of a similar order could be induced in young, 6 week old Dahl-S fed on a high salt diet for 3 weeks, compared to age-matched Dahl-R rats on the same diet. These high plasma ANP concentration were interpreted as a response to hypertension and not a cause of hypertension.

In 1987, Hinko *et al.* (1987) examined the binding characteristics of ANP and the production of cGMP in the kidneys of Dahl-S and Dahl-R rats, on low (0.15%) or high (8%) salt diets. ANP dissociation rates from kidney membranes were similar for Dahl-S and Dahl-R rats on the low salt diet. However, there was a decrease in ANP dissociation rate in the Dahl-R rats with no change in the Dahl-S rats on high salt diets. Receptor density was similar between the two strains of rats on both high and low salt dietary regimes. This group observed that basal renal cGMP production levels were higher in Dahl-S than in Dahl-R rats. However ANP-stimulated renal cGMP generation was similar between Dahl-S and Dahl-R rats independent of the dietary regime. From these results Hinko *et al.* (1987) speculated that the renal receptor of Dahl-R rats which showed salt-induced alterations in ANP dissociation rates was most likely the ANP-C receptor.

The above results indicate that in Dahl-S rats atrial ANP content is increased when compared to Dahl-R rats. This change in ANP content between the two strains of rat is independent of age and dietary regime (a high salt diet suppresses atrial ANP content equally in both strains of rats). Snajdar & Rapp (1985) speculated that the higher atrial ANP content of Dahl-S rats compared to Dahl-R rats may be due to a defect in the ANP secretory mechanism of Dahl-S rats. This would be reflected in decreases in plasma ANP concentration and increases in BP in Dahl-S rats. Onwhochei & Rapp (1989) supported this speculation and showed that a deficiency in ANP release from the atria occurs at an early stage in the development of hypertension and may contribute to the progressive hypertensive state in Dahl-S rats, (as Ferrier *et al.* 1988 and Tunny *et al.* 1986 found in the human). In addition Snajdar & Rapp (1985) speculated

that the hypo-responsiveness of the kidney to ANP in the pre-hypertensive Dahl-S rats would result in increases in BP and increases in plasma ANP concentration. Age and a high salt regime were shown to cause marked increases in BP and also showed increases in plasma ANP concentration in Dahl-S rats when compared to the equivalent Dahl-R rats (Snajdar & Rapp 1986). Therefore, in the Dahl-S rat there may be two genetic defects mediating the effects of ANP; one involving the synthesis, storage and release of ANP and one involving the kidney and its response, Snajdar & Rapp (1985).

1.4.4 Surgically manipulated models for Hypertension

1.4.4.1 The Renal hypertensive rat

Goldblatt and co-workers (Goldblatt *et al.* 1934) were the first to produce a reliable model in dogs, of renovascular hypertension by constriction of a main renal artery following surgery. The initial effect of this constriction is reduced renal arterial pressure, however within a few minutes the systemic arterial pressure begins to rise and continues to do so. Once the systemic arterial pressure reaches a new stable pressure level the renal arterial pressure returns almost to normal (Guyton 1976). Hypertension induced by the constriction of one renal artery while the other kidney is left untouched is referred to as the two-kidney, one-clip, (2K-1C) model. Hypertension induced by the constriction of one renal artery while the other kidney is removed is referred to as the one-kidney, one-clip, (1K-1C) model. The 1K-1C rat at 4 weeks of developed hypertension has normal renin concentrations and volume expansion is present. The 2K-1C rat at an equivalent stage of hypertension has elevated renin concentrations and there is no evidence of volume expansion (Davis 1977). Garcia *et al.* (1987) observed in the 2K-1C and 1K-1C rat models increases in plasma

ANP concentration which correlated well with cardiac weight. They speculated that once the ventricles become hypertrophic they may participate in ANP release. Lattion *et al.* (1990) examined the influence of sodium (for 3wk) in 1K-1C rat model and found that animals receiving a regular sodium diet, (0.27%) showed increases in ANP-specific mRNA in right and left atria compared to salt-restricted, (0.006%) animals. Schiffrin (1989) showed that vascular ANP receptor sites in the 1K-1C and in the 2K-1C rat models, varied inversely with plasma ANP concentration, i.e. an increase in plasma ANP concentration and a decrease in ANP receptor density.

1.4.4.2 The Deoxycorticosterone acetate (DOCA)-salt hypertensive rat

Seyle and colleagues (Seyle *et al.* 1943) were the first to observe that deoxycorticosterone acetate (DOCA), a mineralocorticoid agonist, combined with high salt diet elicited a hypertensive-like syndrome in rats. The (DOCA)-salt hypertensive model is now widely studied to elucidate the precise action of mineralocorticoids in experimental hypertension.

Nuglozeh *et al.* (1990) examined the regulation of receptors for ANP in the renal papilla of the DOCA-salt hypertensive rat. They reported that there was an increase, (141 ± 31 compared to 34 ± 8 fmol/papilla) in receptor density, (only a 125 kDa receptor was present) in the DOCA-salt hypertensive rat compared to controls. However the receptor affinity was similar. The plasma ANP concentration was increased, (408 ± 35 compared to 133 ± 12 pg/ml) with a suppressed plasma renin activity, compared to controls. The production of cGMP in the renal papilla in response to ANP was higher in DOCA-salt rats than in controls. In

density, (in both ANP-B and ANP-C receptors). Thus renal papillary ANP receptors of the DOCA-salt rat are increased and vascular and glomerular ANP receptors are decreased compared to controls. In 1987, Morton *et al.* (1987) and Schiffrin & St-Louis (1987) showed an increase in plasma ANP concentration and a decrease in mesenteric artery vascular ANP receptor density in DOCA-salt rats compared to control rats. In 1989, Schiffrin (1989) also showed that blood vessel vascular ANP receptor sites in the DOCA-salt rat varied inversely with plasma ANP concentration compared to controls.

1.4.5 Summary

In summary, the precise effect of alterations in salt diet on ANP receptor density in the experimental rat models of hypertension is controversial. In general plasma ANP concentration is increased and atrial ANP content decreased, (with the exception of Dahl-S rats where both plasma ANP concentration and atrial ANP content are increased, when compared to Dahl-R rats). In animal models such as Dahl rats there is evidence that a deficiency in ANP secretion early in hypertension (as reported for man by Ferrier *et al.* 1988 and Tunny *et al.* 1986) may promote the development of hypertension. The exact relationship between receptor density, cGMP levels, alterations in salt diet and experimental hypertension in various tissues remains uncertain.

1.4.6 Objectives

Two ANP-specific receptors, most likely the ANP-B and ANP-C receptors have previously been reported in rat sarcolemmal membrane preparations (Rugg *et al.* 1989). As an extension of this work, experiments described in this thesis were aimed at the determination of density (B_{max}), population (ANP-C or ANP-B receptors), molecular size and affinity (K_d)

of ANP-specific receptors in bovine ventricular cardiac sarcolemmal membranes.

Further experimental work presented in this thesis forms part of an on-going investigation in this laboratory to assess the effects of dietary salt regime on plasma ANP concentration, ANP-specific receptor binding kinetics and ANP-specific receptor gene expression in the Dahl hypertension resistant (Dahl-R) and Dahl hypertension sensitive (Dahl-S) rat. Partially purified liver plasma membrane homogenates of male Dahl-R and Dahl-S rats were investigated to assess the effects of resistance and sensitivity to hypertension, in conjunction with a variation in dietary salt regime on population, density, affinity and guanylate cyclase activity of ANP receptors in this tissue. In addition, studies of partially purified liver plasma membrane homogenates of the Wistar rat were carried out to determine the population, density, affinity and guanylate cyclase activity of ANP receptors in this rat.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials.

Rat α-ANP_{1-28} was purchased from Sigma , Poole, Dorset., and also from Biomac, Glasgow University, Glasgow. Des [QSGLG] ANP (4-23)-NH_2 was from Cambridge Research Biochemicals, Cambridge and [Tyr^8] ANP (5-27) was obtained from Peninsula Laboratories, St., Helens. [^{125}I]-ANP (rat α-ANP_{1-28}; specific activity 2200 Ci/mmol) was obtained from NEN/Du Pont (U.K.) Ltd. Stevenage, Herts and and $Na^{125}I$ (specific activity 17Ci/mg; pH 8-10) was obtained from Amersham International plc, Amersham, U.K. Disuccinimydyl suberate (DSS), ethylene glycol *bis* (succinimidylsuccinate) (EGS) and N-hydroxysuccinimidyl-4-azidobenzoate (HSAB) were obtained from Pierce Chemicals U.K., Life Science Labs., Luton, U.K. All other biochemicals were obtained from Sigma, Poole, Dorset, U.K. All general reagents were of analytical grade and supplied by BDH, Poole, Dorset U.K.

The antibody to cGMP was a gift from Dr. P. Hamet (Clinical Research Institute, Montreal, Canada).

Rats (Wistar) were obtained from the University of St. Andrews stocks. These animals were maintained under a 12 h day/night cycle and allowed free access to food.

Rats (Dahl) were obtained from Møllegaard Breeding Center Ltd., Tornbjergvej 40, Ejby, DK 4623 LI. Skensved, Denmark. There were two strains of Dahl rats. One which is resistant to a salt-induced hypertensive state and one which is sensitive to a salt-induced hypertensive state.

Original genetic traits were maintained by brother x sister mating of stock rats and subsequent litters were used for experimental analysis.

At 5 weeks of age the rats were all allowed free access to food (R & M 1 Cube Diet containing 0.8% NaCl) and then after 10 days the rats were split into groups which continued on a normal diet (0.8% NaCl) and groups which were given access to food (modified R & M 1 Cube Diet containing 8% NaCl). The rats were heparinised after a further 5 weeks with 1 ml/Kg of 5000 units/ml i.e. 5000 units/Kg intra peritoneal and killed by decapitation following ether anaesthesia and their tissues removed and frozen in liquid N_2 and stored at - 90 ^{o}C until required.

2.2 Blood Pressure Determination.

Arterial blood pressure was measured in animals from each group i.e. (a) Dahl-R on normal salt-diet (b) Dahl-R on high salt-diet (c) Dahl-S on normal salt-diet and (d) Dahl-S on high salt-diet. Pressures were measured utilising an Apollo Model 179 Blood Pressure Analyser (IITC, Life Sciences USA). Briefly, rats were handled and introduced to the tail cuff apparatus for a period of approximately 2 weeks to reduce any possibility of stress related to the equipment. Rats were then weighed prior to blood pressure monitoring, with a minimum of 3 separate groups of readings taken every week over a 4 week period. (The blood pressure results in Chapter 4 were measured on 3-4 occasions over the last 10 days

of the dietary regime). During the measurements the animals were kept at a constant temperature of 28-29 °C (temperatures any higher tended to cause heat stress).

2.3 Membrane Preparation.

2.3.1 Bovine sarcolemmal membranes.

Bovine hearts were obtained fresh from a local abbatoir and immediately placed in ice cold phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4 and 8.1 mM Na_2HPO_4, pH 7.4). The ventricle was minced finely in a food processor, added to 10 vol. (40 g/200 ml) homogenisation buffer A (1 mM $NaHCO_3$ pH 8.0, containing 0.5 mM dithiothreitol, (DTT), 0.18 mg/ml phenylmethylsulphonylfluoride, (PMSF) and 0.1% bacitracin) and homogenised with a Polytron PT-10 in slurry volumes of 200 ml for 6 x 20 s bursts at setting 5. The resulting homogenates were then diluted 2:1 with salt extraction buffer B (1 mM $NaHCO_3$, 1.2 M KCl, 100 mM $Na_4P_2O_7$, 5 mM $MgCl_2$, 0.5 mM DTT, 0.18 mg/ml PMSF, 0.1% bacitracin, pH 8.0) and stirred slowly for 45 min at 4 °C and then centrifuged at 31,360 g_{av} for 30 min at 4°C, (Beckman J21, Type JA.20 fixed angle rotor). The supernatant was discarded and the pellet resuspended to the initial homogenising volume (200 ml) with 1 mM $NaHCO_3$, 25 mM $Na_4P_2O_7$, 2.5 mM $MgCl_2$, 0.5 mM DTT, 0.18 mg/ml PMSF, 1 μM leupeptin, 0.1 μM pepstatin, 1 μM aprotinin and 0.1% bacitracin, pH 8.0 (buffer C) and recentrifuged at 31,360 g_{av} for 30 min at 4°C. The resulting pellet was resuspended to the initial volume with buffer C which also contained 33% glycerol and then stored in 50 ml aliquots at - 90°C for up to a maximum of 8 weeks. Purified sarcolemmal membrane fractions were obtained as described by Cramb & Dow (1983). Briefly, 100 ml of the glycerol frozen samples were thawed and made up to

320 ml with 1 mM $NaHCO_3$, 25 mM $Na_4P_2O_7$, 2.5 mM $MgCl_2$, 0.5 mM DTT and 0.1% bacitracin and centrifuged at 31,360 g_{av} for 30 min at 4°C. The resulting pellets were resuspended in 30 ml of 5 mM Hepes buffer pH 7.4 containing 0.5 mM DTT to which was added 45 ml 5 mM Hepes pH 7.4 containing 67% (w/v) sucrose and 0.5 mM DTT to give a final concentration of 40% (w/v) sucrose. A 10 ml cushion of 60% (w/v) sucrose in 5 mM Hepes pH 7.4 containing 0.5 mM DTT was overlaid with 18.75 ml of the membrane suspension (40% w/v), 6 ml of 5 mM Hepes pH 7.4 containing of 35% (w/v) sucrose and 0.5 mM DTT and 5 ml of 5 mM Hepes buffer pH 7.4 containing 0.5 mM DTT. The discontinuous sucrose gradients were centrifuged at 100,000 g_{av} for 2 hr at 4°C (Beckman L7, SW28 rotor). The band formed at the 0/35% sucrose interface was collected diluted with 40 ml 5 mM Hepes, pH 7.4 containing 0.5 mM DTT, and centrifuged at 31,360 g_{av} for 30 min at 4°C. The resulting pellet was resuspended in 1-2 ml 5 mM Hepes, pH 7.4, 250 mM sucrose containing 0.5 mM DTT, and stored at -20 °C. The membranes were diluted to use at a final protein concentration of 10-25 µg/ml in radio-receptor and guanylate cyclase assays and 500-700 µg/ml in radio-receptor crosslinking assays.

2.3.2 Partially purified plasma membrane homogenates of rat liver.

The livers from male Dahl-Resistant and male Dahl-Sensitive rats (230-350 g) were removed, weighed and immediately placed in liquid N_2 before subsequent storage at - 90 °C until required. For membrane preparation frozen samples were placed in 80 ml homogenisation buffer (25 mM Tris, 0.25 M sucrose pH 7.4, containing 0.5 mM DTT, 0.18 mg/ml PMSF, 1 µM leupeptin, 0.1 µM pepstatin and 0.1% bacitracin) minced finely with scissors and homogenised with a Polytron PT-10, 6 x 20 s bursts at setting 5. The resulting homogenate was then centrifuged at 666 g_{av} for 10 min

(Beckman J21, Type JA.20 fixed angle rotor). The supernatant was recovered and recentrifuged at 31,360 g_{av} for 30 min at 4°C. The resulting pellet was then resuspended in 20 ml of buffer 1, (25 mM Tris, 0.25 M sucrose, pH 7.4, containing 0.5 mM DTT, 0.18mg/ml), and 40 ml of a 60% (w/v) sucrose solution containing 25 mM Tris pH 7.4, 0.5 mM DTT and 0.18 mg/ml PMSF, to give a final concentration of 43% sucrose. This solution was divided between two 40 ml tubes and overlaid with 10 ml of buffer 1 (8% (w/v) sucrose). The sucrose gradients were centrifuged at 100,000 g_{av} for 2 hr at 4°C (Beckman L7, SW28 rotor). The plasma membrane fraction formed at the 8%/43% interface was collected, diluted with 40 ml buffer 1 and centrifuged at 31,360 g_{av} for 45 min at 4°C. The resulting pellet was resuspended in 2-3 ml buffer 1 and stored at - 20 °C. The membranes were diluted to use at a final protein concentration of 10-25 µg/ml in all assays. Plasma membrane fractions were prepared from the livers of male Wistar rats (250-350 g) as above without initial storage.

2.4 [^{125}I]-ANP Radio-receptor assay.

2.4.1 Bovine sarcolemmal membranes.

Membranes (10-25 µg protein) were incubated over 1 to 6 h at 4 °C, room temperature or 37 °C in 50 mM Tris-HCl, 150 mM NaCl, 0.1 mM EDTA, 5 mM $MgCl_2$, pH 7.4 containing 0.1% BSA, 0.1% bacitracin, 1 µM leupeptin, 0.1 µM pepstatin, 0.1 µM phosphoramidon, 0.1 mM PMSF and 1 µM aprotinin (incubation buffer). The final volume was 200 µl. Total binding was determined in the presence of a fixed concentration of [^{125}I]-ANP (50pM). Specific binding was calculated by subtraction of non-specific binding (determined in the presence of a fixed concentration of [^{125}I]-ANP (50pM) and 0.1µM ANP) from the total binding. Membranes (10-25 µg protein) were also incubated for 1 h at room temperature in incubation

buffer. Binding was determined either in the presence of increasing concentrations of [^{125}I]-ANP (2-800 pM), in the presence or absence of unlabelled ANP (1 μM), or in the presence of a fixed concentration of [^{125}I]-ANP (50 pM) and increasing concentrations of unlabelled ANP, des [QSGLG] ANP (4-23)-NH_2 or [Tyr^8]-ANP (5-27) (1 pM - 10 μM). Incubations were terminated by the addition of 2 ml of ice cold wash buffer (50 mM Tris, 150 mM NaCl pH 7.4 at 4 ^oC) containing 1% BSA. Bound [^{125}I]-ANP was separated from free by rapid filtration through Whatman GF/C filters followed by three washes with 9 ml of wash buffer. To reduce non-specific binding of the radiolabel the filters were pre-soaked in 0.3% polyethyleneimine (PEI) for at least 12 h prior to use, (Brunks *et al.* 1983). Incubations were staggered and the total filtration time including the washes was less than 20 s. Radioactivity was determined in a Packard Prias gamma counter. Scatchard analysis was determined by the ENZFITTER software package (a non-linear regression data analysis programmme) for the IBM computer, Leatherbarrow (1987).

2.4.2 Partially purified plasma membrane homogenates of rat liver.

The binding assay was carried out as described above for the bovine sarcolemmal membranes.

2.5 [^{125}I]-ANP Receptor crosslinking assay.

2.5.1 Bovine sarcolemmal membranes

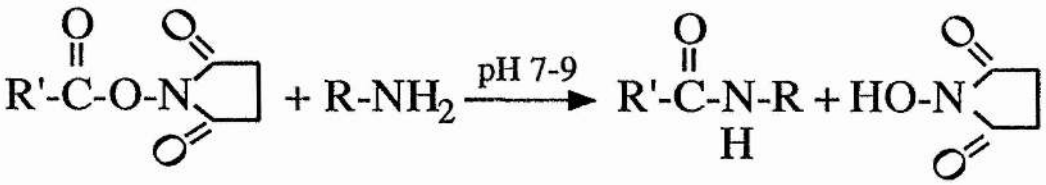
Sarcolemmal membranes (700 μg protein) were incubated with 50 pM [^{125}I]-ANP in the presence or absence of various concentrations of unlabelled ANP or des [QSGLG] ANP (4-23)-NH_2. for 1 h at room temperature in 50 mM Tris/Hepes, 0.1 mM EDTA, 5 mM $MgCl_2$, pH 7.4 containing 0.1% BSA, 0.1% bacitracin. The incubation buffer for binding

in these crosslinking experiments initially contained 50 mM Hepes buffer in the place of Tris buffer, since Tris buffer is reported to interfere with crosslinking (Pierce 1988). However the subsequent washing protocol used prior to crosslinking eliminated any interference from the Tris and results with both Tris and Hepes buffers were found to be the same. The final volume was 2 ml. Incubations were terminated by the addition of 3 ml of ice-cold 50 mM K_2HPO_4/KH_2PO_4 pH 7.4 and the membranes were pelleted by centrifugation at 59,200 g_{av} for 15 min at 4 °C. The pellet was washed once with 5 ml of 50 mM K_2HPO_4/KH_2PO_4 pH 7.4 and recentrifuged at 59,200 g_{av} for 15 min at 4 °C. The membrane pellet was then resuspended in 2 ml of 50 mM K_2HPO_4/KH_2PO_4 pH 7.4 containing 0.1 mM disuccinimidyl suberate (DSS) and incubated for 40 min on ice. The crosslinking reaction was terminated by the addition of 1 M ammonium acetate to a final concentration of 50 mM and diluted to 5 ml with 50 mM K_2HPO_4/KH_2PO_4 pH 7.4 and centrifuged at 59,200 g_{av} for 15 min at 4 °C. The pelleted membranes were resuspended in 5 ml of 62.5 mM Tris/HCl pH 6.8 and recentrifuged at 59,200 g_{av} for 15 min at 4 °C. This final pellet was used directly as a sample in SDS-PAGE.

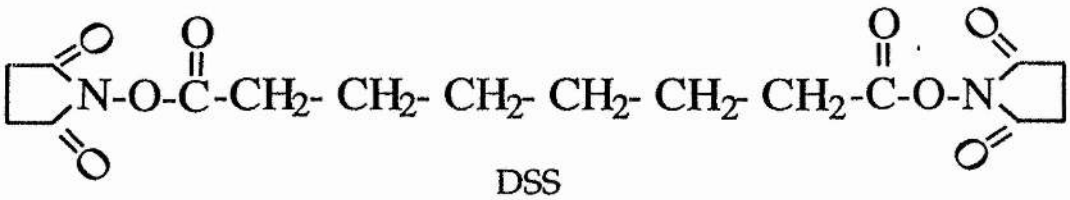
N-hydroxysuccinimide (NHS) homobifunctional crosslinkers such as DSS, react with primary amine functions of epsilon (ε) amine groups on lysine or available N-terminal amines. At pH 7-9, the amino group on a particular ligand such as [^{125}I]-ANP undergoes nucleophilic attack of the NHS-ester to form a stable amide bond and release N-hydroxysuccinimide as a by-product (see fig. 2.1).

Figure 2.1

NHS Ester Reaction Scheme and the Structure of DSS



NHS Ester Reaction Scheme



DSS

M.W. 368.35

Length 11.4 Å

2.5.2 Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis.

Electrophoresis was performed using a 7.5% separating gel and the discontinuous buffer system of Laemmli (1970). Briefly, 27.2 ml of a 30% (w/v) stock acrylamide solution containing 2.7% (w/w) of the bis-acrylamide crosslinker was diluted to 7.5% acrylamide with 20 ml of 4 x separating gel buffer (1.5 M Tris-HCl, pH 8.8 containing 0.4% SDS) and 32.8 ml of distilled water, total volume of 80 ml. The crosslinking agents, ammonium persulphate (320 µl of 10% solution) and TEMED (120 µl) were added and the gel solution poured between two (16 x 16.8 cm) glass plates separated by 3mm spacers and the solution allowed to polymerize. A stacking gel of 4.5% was also prepared; 3 ml of a 30% (w/v) stock acrylamide containing 2.7% (w/w) of the bis-acrylamide crosslinker was diluted to 4.5% acrylamide with 5 ml of 4 x stacking gel buffer (0.5M Tris-HCl, pH 6.8 containing 0.4% SDS) and 12 ml of distilled water, total volume of 20 ml. The crosslinking agents, ammonium persulphate (160 µl of 10% solution) and TEMED (60 µl) were added and the gel solution layered on top of the 7.5% polymerized gel. A 3 mm thick, 20 toothed comb was placed in the 4.5% acrylamide solution and polymerization allowed to occur. The completed gel system was clamped into the electrophoresis apparatus and reservoir buffer (0.192 M Glycine, 0.025 M Tris, 0.1% SDS, pH 8.3) added to the upper and lower chambers. The comb was removed and protein samples were loaded into the wells. The gel system was then linked to a power pack and run under a constant voltage of 200 V (running time 2-3 h). Gels were finally removed from the apparatus stained with (Coomassie Blue-R 0.1% w/v, methanol 25% v/v, acetic acid 10% v/v and H_2O 65% v/v) and destained with (methanol 50% v/v, acetic acid 10% v/v and H_2O 40% v/v).

The relative mobility (R_f) of a protein which is logarithmetically related to the molecular weight can be calculated by dividing the distance of the protein migration by the distance of the tracking dye migration. (A typical calibration curve for SDS-PAGE molecular weight markers is shown in figure 2.2 b. Estimates of the molecular weight of unknown proteins can be made from the calibration curve).

2.5.3 SDS-PAGE of bovine sarcolemmal membranes.

The resulting pellets from the cross linking reaction were suspended in 200 µl distilled water and diluted 1 : 1 with double strength sample buffer (125 mM Tris-HCl, 4.6% SDS, 8 M Urea, 0.002% bromophenol blue, pH 6.8) in the presence or in the absence of 10% (v/v) β-mercaptoethanol and boiled for 5 min. The equivalent of 230 µg of protein was loaded onto each lane of the gel and run as above. Gels were stained and destained as above. Gels were then stored in Philips 18 x 24 cm Ultra cassettes at - 20 °C for 7-14 days with Amersham Hyper film-MP, preflashed to increase the sensitivity of the film (the flash gun was set at a power ratio of $^1/_{16}$ and used at a distance of 6 feet in the presence of a Kodak No. 22 wratten gelatin filter). Autoradiographs were developed after 7-14 days. Autoradiographs and SDS-PAGE gels were finally scanned utilising a Shimadzu Model CS-9000 Dual-Wavelength Flying-Spot Scanner, (Shimadzu Corporation, Kyoto, Japan). Autoradiographs and SDS-PAGE gel standard molecular weight markers were scanned at wavelengths of 650 nm and 590 nm respectively (a representative scan of an SDS-PAGE gel with molecular weight markers of 200, 116, 97.4, 66, 45 and 29 kDa is shown in fig. 2.2 a). The area under the peak is representative of the intensity of Coomassie Blue-R stain on the gel and reflects the concentrations of the individual proteins loaded onto the gel.

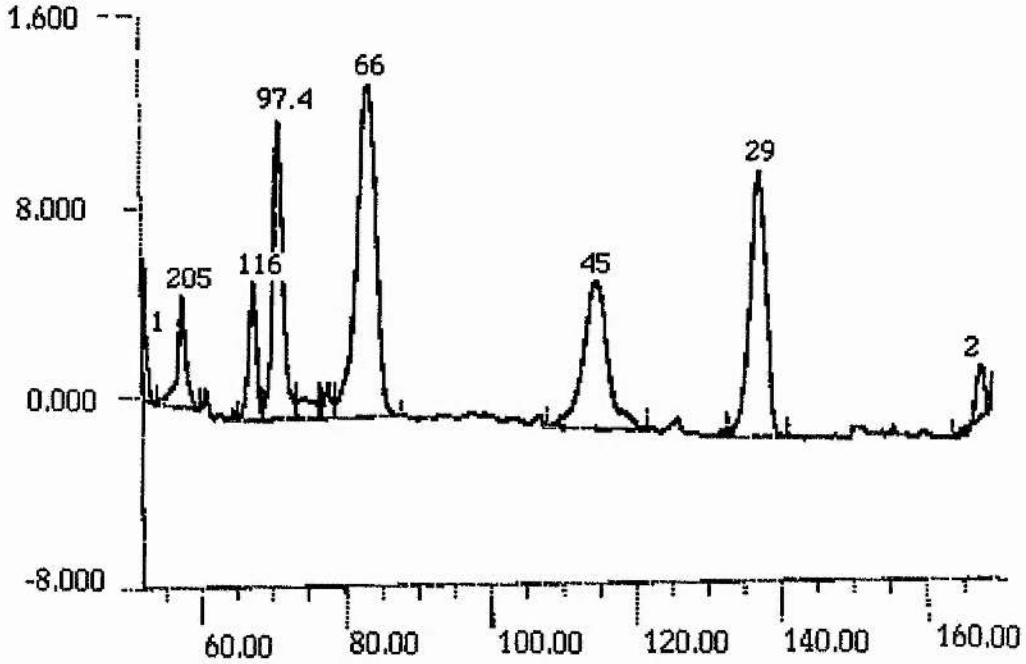
Figure 2.2

A representative scan of SDS-PAGE molecular weight standard markers and a typical calibration curve.

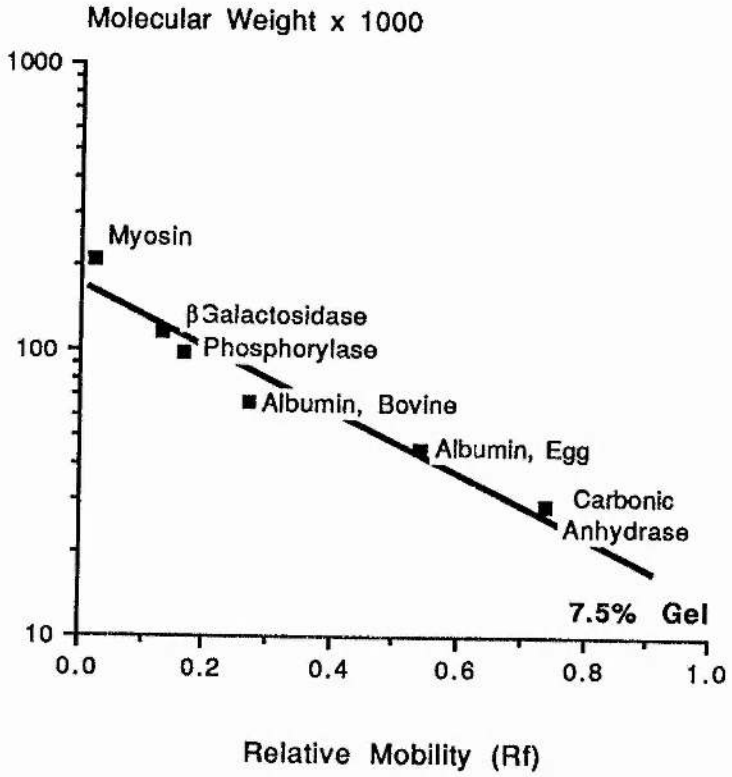
a) A representative scan of SDS-PAGE molecular weight standards from the scanning densitometer. The indicated peaks are the appropriate molecular weight standard positions with [1] indicating the start of the resolving gel and [2] indicating the position of the dye front.

b) A calibration curve of the the relative mobility of SDS-PAGE molecular weight standards. The molecular weight of unknown proteins can be calculated from this curve, (line fitted by eye).

a


1.600
8.000
0.000
-8.000
66
97.4
29
205
116
45
1
2
60.00
80.00
100.00
120.00
140.00
160.00

b


Molecular Weight x 1000
1000
100
10
Myosin
βGalactosidase
Phosphorylase
Albumin, Bovine
Albumin, Egg
Carbonic
Anhydrase
7.5% Gel
0.0
0.2
0.4
0.6
0.8
1.0
Relative Mobility (Rf)

2.5.4 [^{125}I]-ANP crosslinking assay and SDS-PAGE of partially purified plasma membranes isolated from rat liver.

The [^{125}I]-ANP receptor crosslinking assay and SDS-PAGE was carried out as described above for the bovine sarcolemmal membranes.

2.6 Measurement of Guanylate Cyclase Activity.

2.6.1 Guanylate cyclase assay.

Guanylate cyclase activity was measured in bovine cardiac sarcolemmal membranes and rat liver plasma membranes as follows. Membranes were incubated in the presence of 50 mM triethanolamine, pH 7.4, 1 mM GTP, 3 mM $MnCl_2$, 2 mM IBMX, 10 mM theophylline, 0.1 mg/ml creatine phosphokinase and 5 mM creatine phosphate for 20 min at 37 °C, in a final assay volume of 100 µl, which also contained ANP, des-ANP, Tyr^8-ANP (1 pM- 1 µM) or buffer, (for basal levels). Incubations were initiated by the addition of GTP and the final protein concentration was 10 µg/tube for both membrane preparations. Incubations were terminated by the addition of 1 ml of 30 mM EDTA at greater than 90°C and the solution assayed for cGMP by radioimmunoassay. To ensure there was no interference of reagents with the subsequent cGMP assay, zero time points were prepared which contained all of the reagents and the membranes but were not incubated.

2.6.2 Radioimmunoassay for cGMP.

cGMP was determined by radioimmunoassay as described by Richman *et al.* (1980). Duplicate 100 µl samples of buffer, standard or unknown were placed in Sterlin RT 30 tubes followed by 100 µl of [^{125}I]-ScGMP-TME (10,000-15,000 cpm/100 µl, see below) and 250 µl of the cGMP antibody, (diluted 1 : 45,000). The [^{125}I]-ScGMP-TME and the cGMP Ab were both

prepared in 50 mM sodium acetate pH 4.75 containing 0.5% BSA, as were the standards. The reagents were allowed to equilibrate by incubation overnight (15 - 24 h) at 4 °C. The Ab was precipitated by the addition of 2 ml of cold 96% ethanol. The tubes were vortexed and allowed to stand at room temperature for 30 min. The resulting precipitate was pelleted at 1,500 g for 30 min at 4 °C (Fisons Coolspin Centrifuge) and the supernatant removed by aspiration. Radioactivity of the pellet was determined by a Packard Prias gamma counter.

For each experiment a standard curve in the range of 0.0625-8 pmol/100 µl cGMP was prepared. Non-specific binding was defined as the radioactivity bound in the presence of excess cGMP (20 nmol) and was subtracted from all values. Results were expressed as counts bound in the absence (zero standard) of cGMP (Co) divided by the counts bound in the presence of cGMP (Cx). A plot of $^{Co}/_{Cx}$ against cGMP concentration produced a standard curve from which the amount of cGMP in each sample (unknown) was estimated, (see fig 2.3 a).

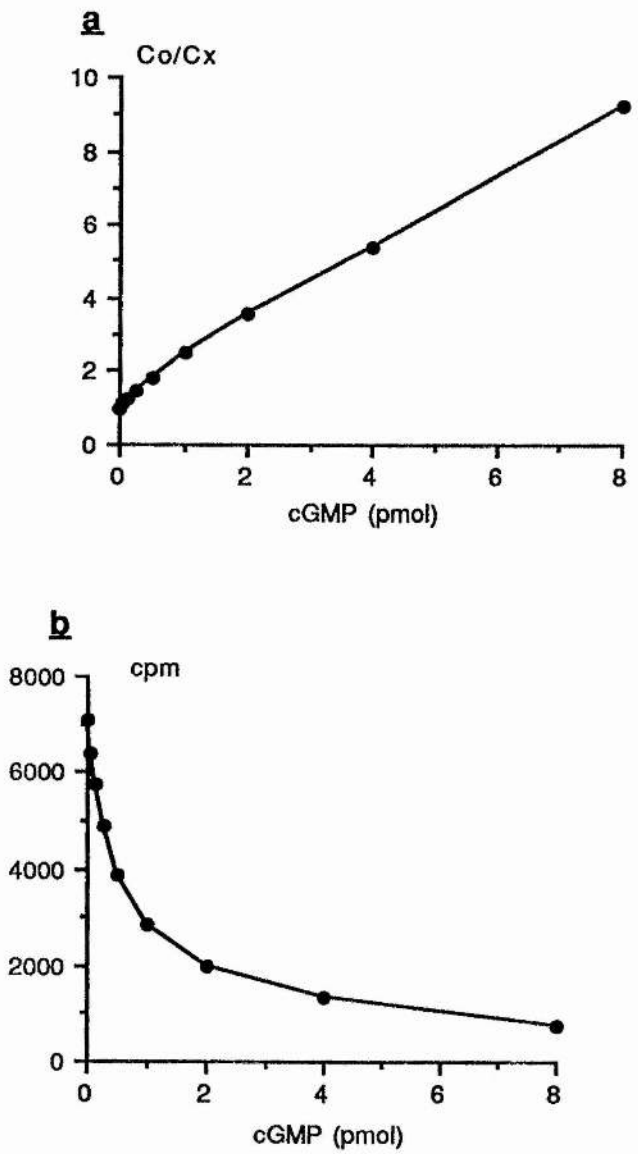
2.6.3 Preparation of [^{125}I]-Tyrosine Methyl Ester Succinyl-cGMP.

Succinyl-cGMP tyrosine methyl ester (ScGMP-TME) was labelled with [^{125}I] according to the method described by Richman *et al.* (1980). Briefly, 20 µl of ScGMP-TME (2 µg) and 10 µl of Na ^{125}I (approx. 1 mCi) were placed in a 1.5 ml microfuge tube and the reaction initiated by the addition of 20 µl chloramine-T (1 mg/ml in 50 mM potassium phosphate buffer pH 7.4). The reaction was terminated after 45 s by the addition of 50 µl of sodium metabisulphite (1 mg/ml in 50 mM potassium phosphate buffer pH 7.4). Sodium iodide (100 µl of 5 mM) was then added to reduce the specific activity of the unreacted [^{125}I] and to dilute the ionic strength to less than

Figure 2.3

Measurement of cGMP by Radioimmunoassay (RIA)

(a) A representative standard curve for the assay of cGMP. Each point is the mean ± S.D. of three determinations. (b) A plot of radioactivity bound against cGMP concentration. (Lines fitted by eye, errors less than 1% of each value).



250 mM. The reaction mix was layered on top of a QAE-25 Sephadex column which had been pre-equilibrated with 50 mM ammonium formate, pH 6 at 4 °C. The reaction mix was allowed to run into the column and was then washed with approximately 300 µl of 250 mM ammonium formate, pH 6 at 4 °C. The [^{125}I]-Sc-cGMP was eluted with 250 mM ammonium formate, pH 6 at 4 °C, at a flow rate of 1 ml/min at 4 °C. Fractions of 2 ml were collected and 5 µl aliquots from these were counted for radioactivity. Fractions corresponding to peaks in radioactivity were diluted to 15,000 cpm/100 µl and assayed for their binding to the cGMP Ab. The fractions containing the highest binding were pooled and stored in 200 µl aliquots at - 20 °C, (see fig 2.4).

2.7 Protein Assay.

Protein concentrations were determined using Bradfords protein estimation solution, (Bradford 1976). Bradfords solution was prepared as follows; 100 mg of Coomassie Blue-G250 was dissolved in 50 ml of 95% ethanol, 100 ml of 85% (w/v) orthophosphoric acid was added and the solution made up to 1 l with distilled water. The final solution was ready for use after filtration with Whatman No.1 filter paper. The protein standards, samples or buffer (100 µl) were added to 5 ml of Bradfords solution, mixed and allowed to stand at room temperature for 2 min. The absorbance was then measured at 595 nm in a Philips PU 8620 spectrophotometer. Bovine serum albumin was used as a standard, (BSA at 1 mg/ml, measured at 280 nm should give a value of 0.67), with sample buffer blanks and the assay was linear over the range 10-100 µg of protein. The protein concentration of the sample was determined from the regression line of the standard curve for BSA, (see fig. 2.5).

Figure 2.4

Purification of [^{125}I]-ScGMP-TME

Profile of radioactivity eluted from a QAE-25 Sephadex column. Fractions from peaks 1, 2 and 3 were assayed for cGMP antibody binding. The dashed area represents the fractions which were pooled to give [^{125}I]-ScGMP-TME for use in the cGMP assay.

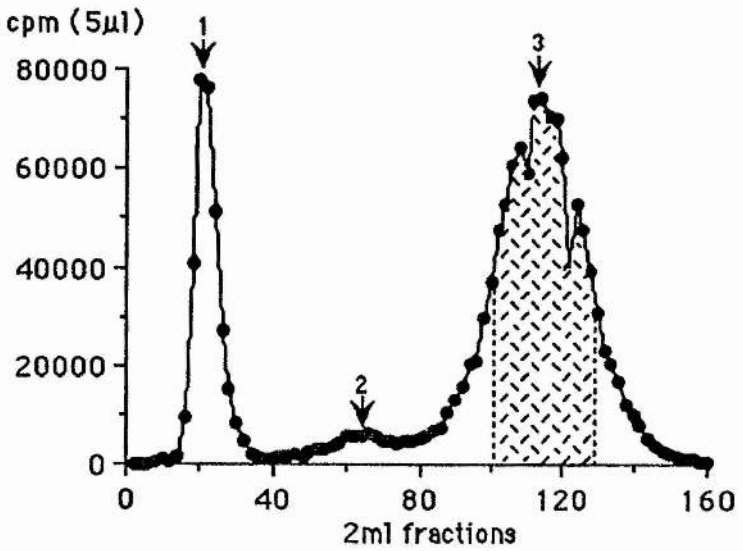
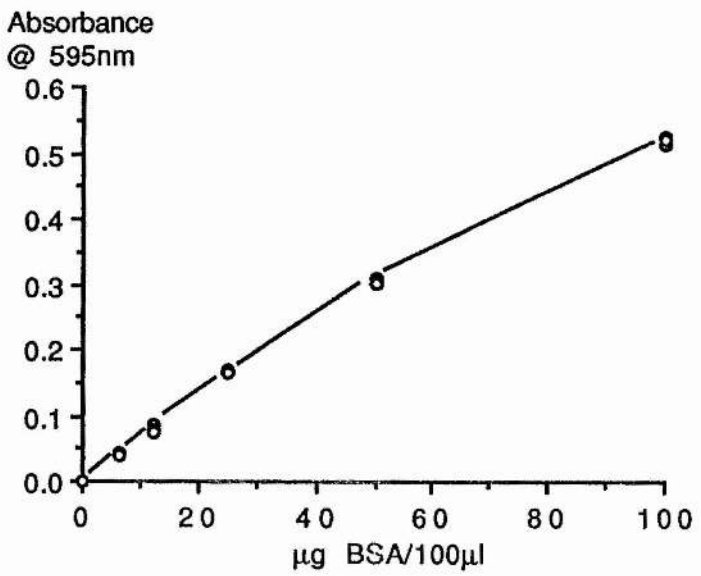


Figure 2.5

Measurement of protein using Bradford's protein assay.

A representative standard curve for the Bradford's protein assay. Each point represents a separate determination. Bovine serum albumin was used as the standard. The absorbance was measured at 595 nm.



2.8 Statistics

The data in this thesis is presented as the mean ± S.D. (standard deviation), with the exception of the guanylate cyclase assays where the data is presented as the mean ± S.E.M. (standard error of the mean). Where there are no error bars indicated on graphs, the errors were less than 1% of the mean value. Graphic lines were fitted by eye with the exception of the Scatchard analysis where the lines were fitted by the ENZFITTER programmme for IBM p.c. Leatherbarrow (1987).

Statistical analysis in Chapter 4 was determined using the Student's unpaired t-test. The relative degrees of freedom and the probability values for each appropriate test are listed in Chapter 4.

CHAPTER 3

RESULTS AND DISCUSSION OF BOVINE VENTRICULAR SARCOLEMMAL MEMBRANE EXPERIMENTS

3.1 Introduction

Bovine ventricular sarcolemmal membranes (BS membranes) were prepared as described in the Materials and Methods. Radio-receptor binding, radio-receptor crosslinking and guanylate cyclase assays, with ANP and ANP analogues were carried out on these membrane preparations. From these assays the density (B_{max}), population (ANP-C or ANP-B receptors) and affinity (K_d) of ANP specific receptors in bovine ventricular sarcolemmal membranes was determined.

3.2 [^{125}I]-ANP Radio-receptor Binding

3.2.1 Displacement of [^{125}I]-ANP by ANP

Incubation of [^{125}I]-ANP with BS membranes at 4 ^{o}C (see fig. 3.1), 22 ^{o}C (see fig. 3.2) and 37 ^{o}C (see fig. 3.3) resulted in time-dependent increases in radioactivity. Specific binding, defined as that binding not displaced by 0.1 μM ANP, reached a steady state within 5 hr, 60 min and 30 min for experiments performed at 4 ^{o}C, 22 ^{o}C and 37 ^{o}C respectively. The times required for half maximal specific binding ($t_{1/2}$) at 4 ^{o}C, 22 ^{o}C, 37 ^{o}C were 75 min, 12 min and 10 min respectively. Subsequent experiments were performed for 60 min at 22 ^{o}C. To assess the affinity and total density of [^{125}I]-ANP binding sites in BS membranes a saturation binding curve was constructed from three BS membrane preparations which had been previously incubated with 0.005% TX-100 for 15 min at 4 ^{o}C (see fig. 3.4 a). The presence of a maximum concentration of [^{125}I]-ANP (1 nM) was not sufficient to saturate all of the ANP receptor population. A Scatchard plot

Figure 3.1

Time course for the binding of [^{125}I]-ANP to bovine ventricular sarcolemmal membranes at 4 °C.

[^{125}I]-ANP (50 pM) was incubated with BS membranes (25 µg/100 µl) at 4 °C, in the absence (O-O, total) and the presence (□-□, NSB) of 0.1 µM ANP. At the indicated times tube contents were filtered through Whatman GF/C filters as described in the Materials and Methods. The specific binding (■-■) was calculated by subtraction of the NSB form the total binding. Each point represents the mean of at least 3 determinations (errors less than 1% of each value), from a single preparation.

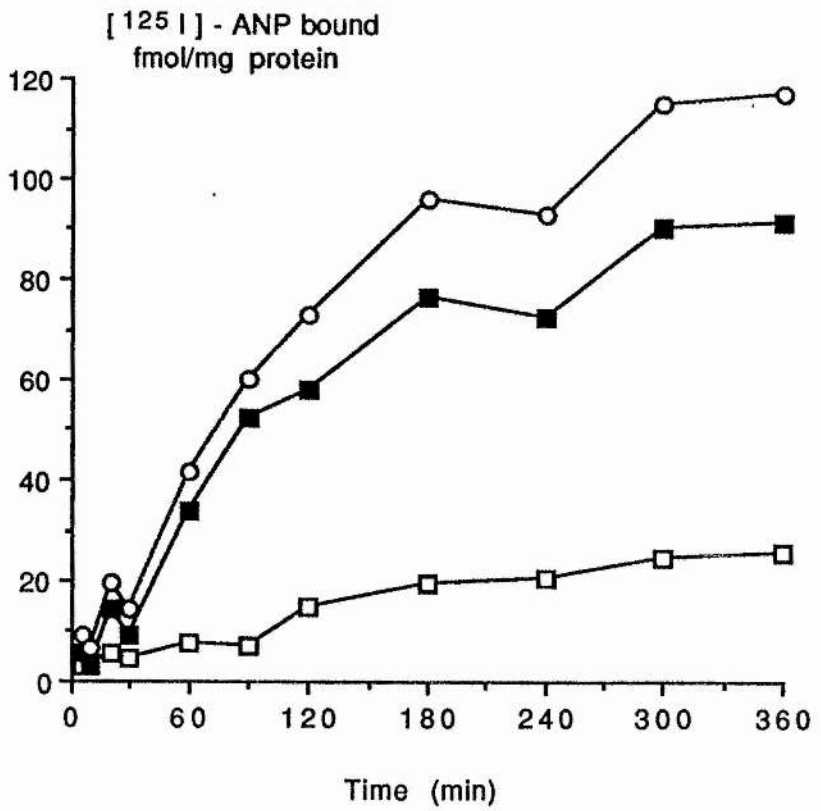


Figure 3.2

Time course for the binding of [^{125}I]-ANP to bovine ventricular sarcolemmal membranes at room temperature.

[^{125}I]-ANP (50 pM) was incubated with BS membranes (25 µg/100 µl) at room temperature in the absence (O-O, total) and the presence (□-□, NSB) of 0.1 µM ANP. At the indicated times tube contents were filtered through Whatman GF/C filters as described in the Materials and Methods. The specific binding (■-■) was calculated by subtraction of the NSB form the total binding. Each point represents the mean of at least 3 determinations (errors less than 1% of each value), from a single preparation.

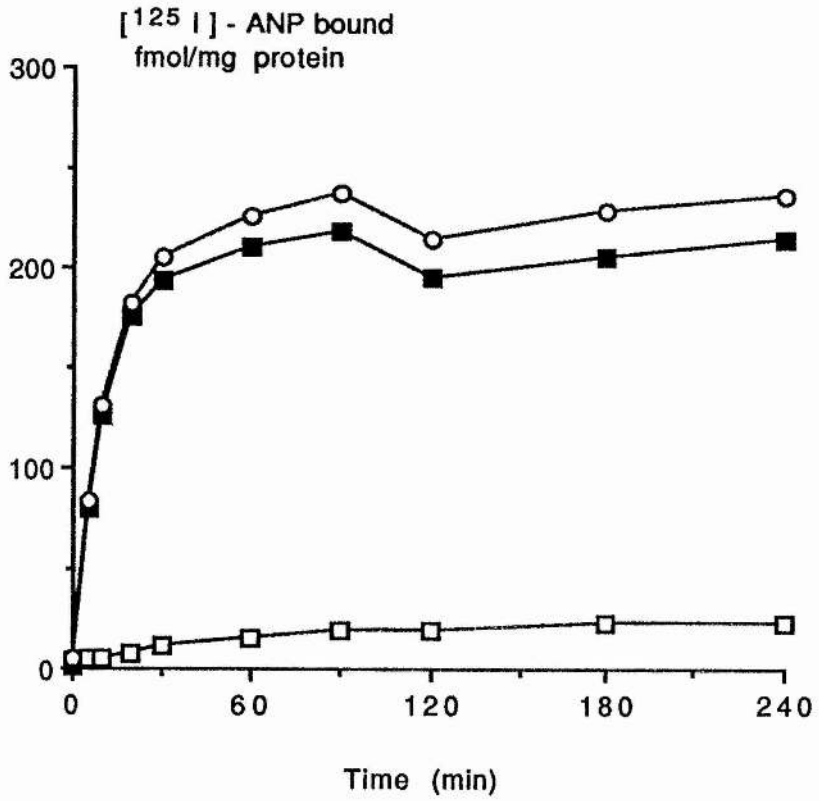
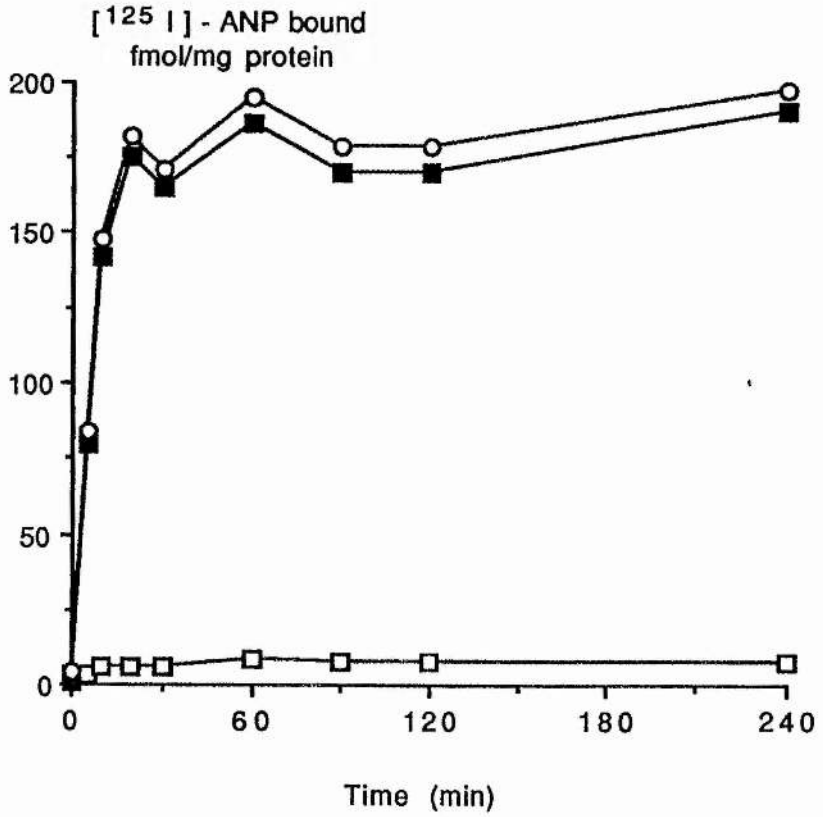


Figure 3.3

Time course for the binding of [^{125}I]-ANP to bovine ventricular sarcolemmal membranes at 37 °C.

[^{125}I]-ANP (50 pM) was incubated with BS membranes (25 μg/100 μl) at 37 °C, in the absence (O-O, total) and the presence (□-□, NSB) of 0.1μM ANP. At the indicated times tube contents were filtered through Whatman GF/C filters as described in the Materials and Methods. The specific binding (■-■) was calculated by subtraction of the NSB form the total binding. Each point represents the mean of at least 3 determinations (errors less than 1% of each value), from a single preparation.



of the data (see fig. 3.4 b) indicated the presence of only one receptor site with a K_d of 43.6 ± 8.28 pM and a B_{max} of 48.5 ± 2.7 fmol/mg protein. These results however do not rule out the possibility of other lower affinity sites in these membranes since saturation of all the binding sites was not achieved. (Due to expense, this experiment could not include any concentrations of [^{125}I]-ANP higher than 1 nM). The displacement of [^{125}I]-ANP (50 pM) by unlabelled ANP was measured in three separate preparations (see fig. 3.5 a). The data from these experiments was combined and [^{125}I]-ANP bound was expressed as a percentage of the maximum specific binding. The concentration of ANP required to produce a 50% inhibition of binding (IC_{50}) was determined as 140 ± 60 pM. The displacement of [^{125}I]-ANP (50 pM) by unlabelled ANP was also investigated in BS membranes which had been previously solubilised with 0.1% TX-100 (see fig. 3.5 b). This data provided an IC_{50} value of 40 pM. Solubilisation of the membranes did not increase the total specific binding of [^{125}I]-ANP, (45.92 ± 2.82 fmol/mg (n = 1) as opposed to 59.22 ± 35.99 fmol/mg (n = 3) in the absence of TX-100 treatment), thus suggesting that detergent action on the membranes did not allow [^{125}I]-ANP access to an increased number of receptor sites. The displacement of [^{125}I]-ANP (50 pM) by des-ANP and Tyr^{8}-ANP was also examined in separate BS membrane preparations (see figs. 3.5 c and d). The IC_{50} values for these analogues were determined as 1 μM and 1 nM respectively. Using the equation,

$$Kd = IC_{50} / 1 + \left([HOT] / Kd\ (HOT)\right)$$

K_d is the dissociation constant for unlabelled ANP

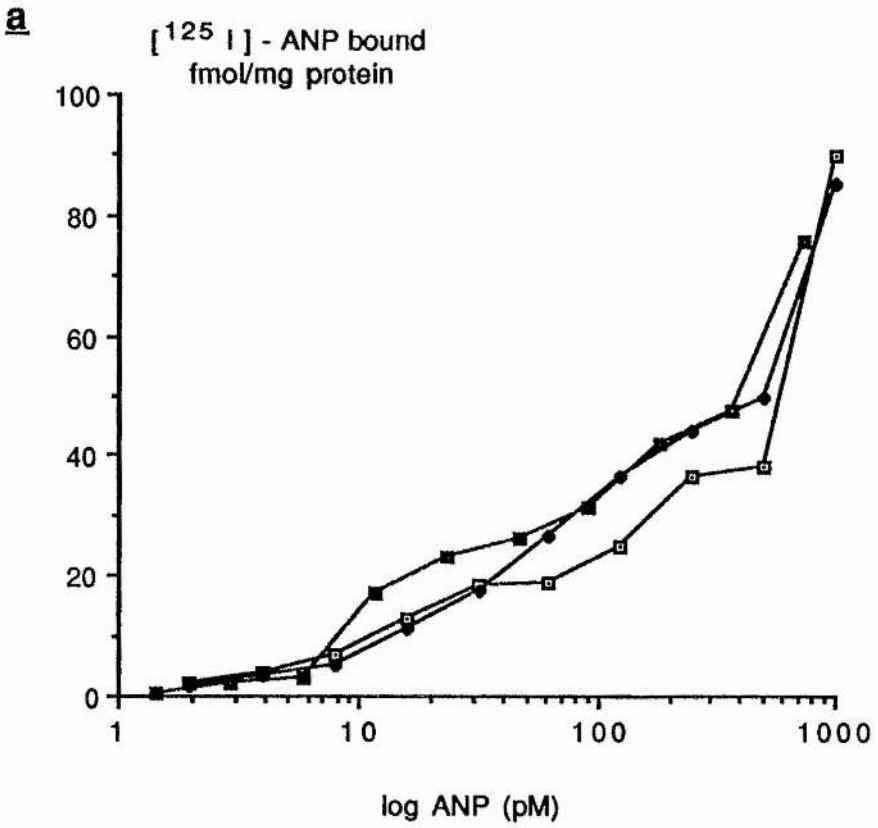
IC_{50} is the concentration of ANP resulting in half maximum displacement of radioligand

Figure 3.4

Saturation curve for [^{125}I]-ANP binding to bovine ventricular sarcolemmal membranes. (Prior incubation with 0.005% TX-100).

(a) BS membranes were incubated for 1 hr at 22 °C, with increasing concentrations of [^{125}I]-ANP. NSB was determined in the presence of 1 μM ANP for each concentration of [^{125}I]-ANP; specific binding was then calculated by subtraction of the NSB from the total binding. Each point represents the mean of at least 3 determinations (errors less than 1% of each value), from three separate preparations.

(b) Scatchard plot of the combined data shown in (a) calculated using the ENZFITTER programme on IBM Computer.


a
[125 I] - ANP bound
fmol/mg protein
100
80
60
40
20
0
1
10
100
1000
log ANP (pM)

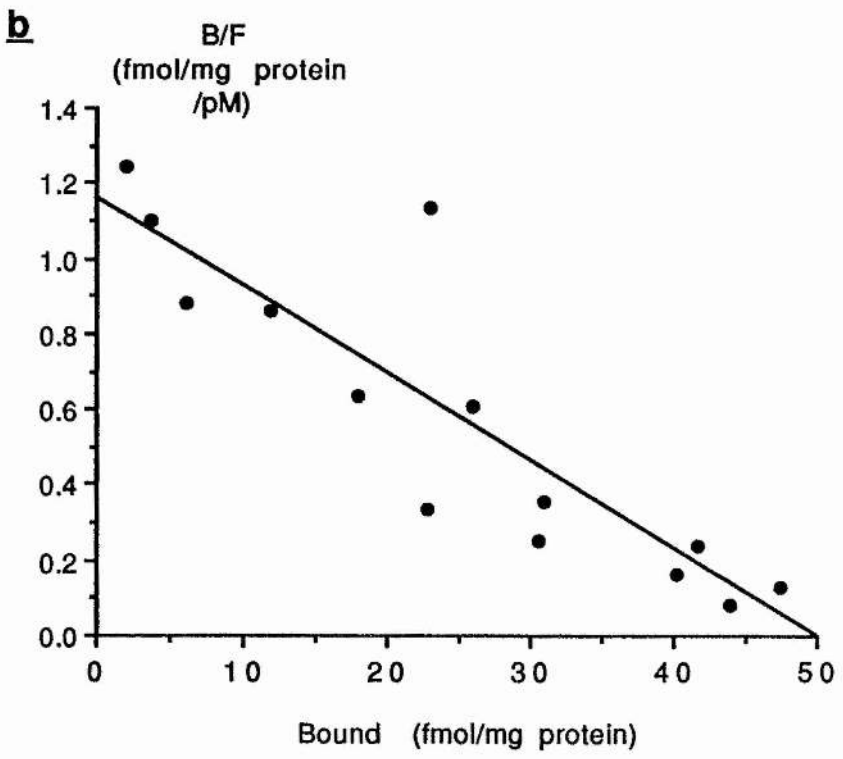
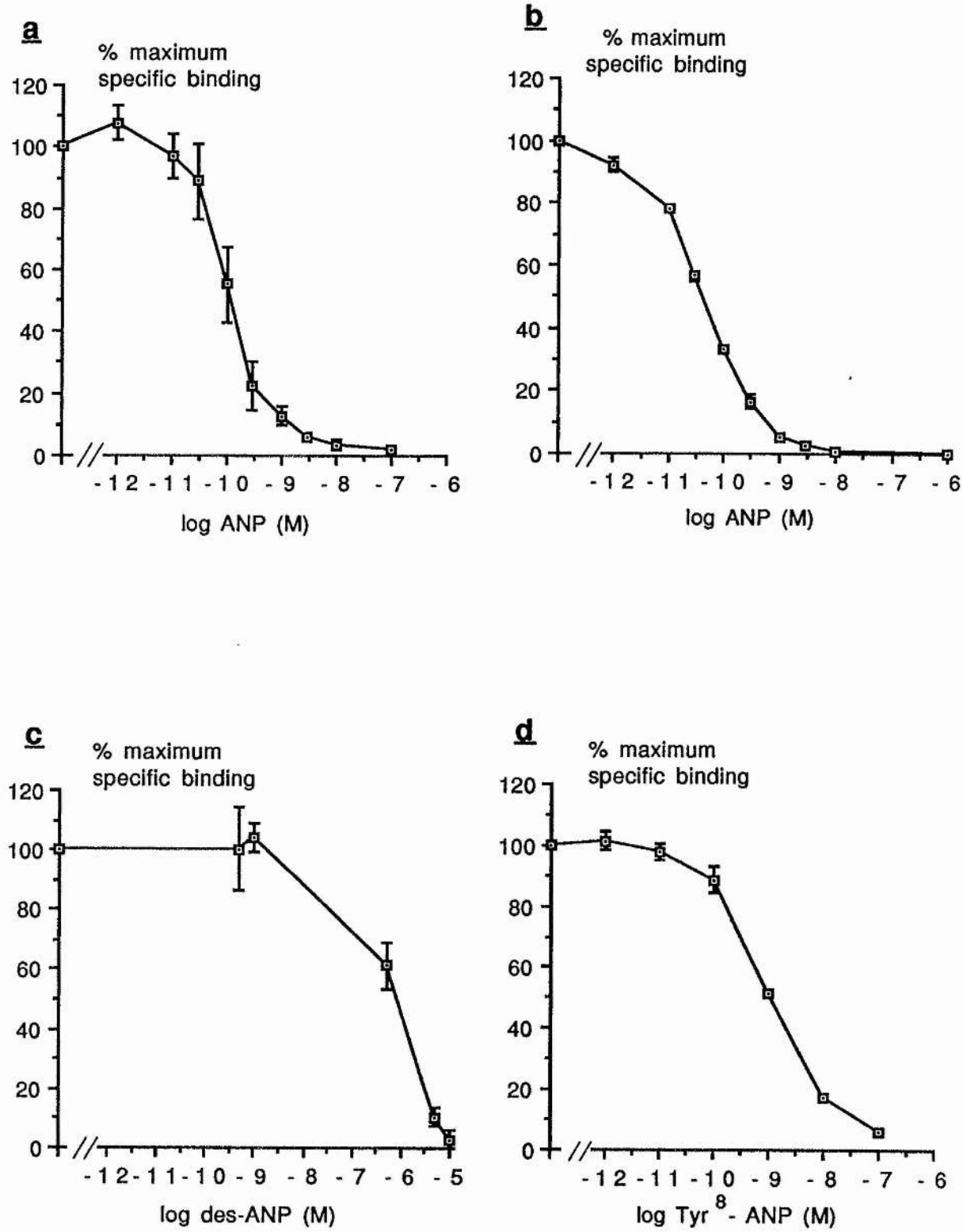

b
B/F
(fmol/mg protein
/pM)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Bound (fmol/mg protein)

Figure 3.5

Inhibition of [^{125}I]-ANP binding in bovine ventricular sarcolemmal membranes by ANP, des-ANP and Tyr^{8}-ANP.

Dose response curves for the displacement of [^{125}I]-ANP binding form BS membranes (25 μg/100 μl) by (a) ANP, (b) ANP in solubilised BS membranes, (c) des-ANP and (d) Tyr^{8}-ANP are shown. NSB was determined in the presence of 1 μM ANP for each curve and specific binding was then calculated by subtraction of the NSB form the total binding. Values are expressed as % of the maximum specific binding of [^{125}I]-ANP and each point represents the mean of at least 3 determinations ± S.D. Individual experiments were performed on separate preparations.


a
% maximum
specific binding
120
100
80
60
40
20
0
-12 -11 -10 -9 -8 -7 -6
log ANP (M)
b
% maximum
specific binding
120
100
80
60
40
20
0
-12 -11 -10 -9 -8 -7 -6
log ANP (M)
c
% maximum
specific binding
120
100
80
60
40
20
0
-12 -11 -10 -9 -8 -7 -6 -5
log des-ANP (M)
d
% maximum
specific binding
120
100
80
60
40
20
0
-12 -11 -10 -9 -8 -7 -6
log Tyr 8 - ANP (M)

[HOT] is the concentration of [^{125}I]-ANP used in the dissociation experiment

K_d (HOT) is the dissociation constant for [^{125}I]-ANP calculated from the saturation experiments

the K_d values of the above experiments were calculated (see table 3.1). The K_d for ANP binding in BS membranes was calculated to be 65 ± 27.85 pM. Solubilisation of the membranes with 0.1% TX-100 did not have any effect on the B_{max} but did decrease the K_d for ANP by approximately 3-fold to 18.7 pM. The K_d for des-ANP in non-solubilised BS membranes was calculated to be 486 nM, approximately 7000-fold higher than the K_d value for ANP and the calculated K_d value for Tyr^8-ANP was approximately 7-fold higher than the value for ANP at 466 pM.

3.2.2 Displacement of [^{125}I]-ANP by BNP

Hirata *et al.* (1988) have previously shown that BNP can bind to the same receptor site as ANP. The displacement of [^{125}I]-ANP (50 pM) by unlabelled BNP was therefore examined in a BS membrane preparations (see fig. 3.6). BNP was found to displace [^{125}I]-ANP in BS membranes with an IC_{50} value of 400 pM.

3.3 [^{125}I]-ANP Receptor crosslinking experiments.

Initial experiments were performed to assess the ability of DSS to crosslink [^{125}I]-ANP to its receptor site(s) in BS membranes for 1hr at 22°C (see fig. 3.7). These early experiments indicated the presence of two molecular weight receptor binding sites for ANP in BS membranes. Scans of autoradiographs revealed that these sites had molecular weights of 60 kDa and 120 kDa. Large amounts of radiolabel were detected at the top of these autoradiographs indicating that crosslinked protein material was not

Table 3.1

Estimated IC_{50} values and calculated K_d values for the dissociation of [^{125}I]-ANP by ANP, des-ANP and Tyr^8-ANP in BS membranes.

The IC_{50} values were estimated from Figs. 3.5a-3.5d

Peptide	Estimated IC_{50} value	Calculated K_d value
ANP[1]	140 ± 60 pM	65 ± 28.75 pM
ANP[2]	40 pM	18.7 pM
des-ANP	1 μM	486 nM
Tyr^8-ANP	1 nM	466 pM

[1] Dissociation of [^{125}I]-ANP in BS membranes by ANP.

[2] Dissociation of [^{125}I]-ANP in BS membranes by ANP, previously incubated for 15min with 0.1% TX-100.

Figure 3.6

Inhibition of [^{125}I]-ANP binding in bovine ventricular sarcolemmal membranes by BNP.

A representative dose response curve for the displacement of [^{125}I]-ANP binding form BS membranes (25 µg/100 µl) by BNP is shown. Non-specific binding was determined in the presence of 0.1 µM BNP and specific binding was then calculated by subtraction of the NSB from the total binding. Values are expressed as % of the maximum specific binding of [^{125}I]-ANP and each point represents the mean of at least 3 determinations ± S.D. from an individual preparation.

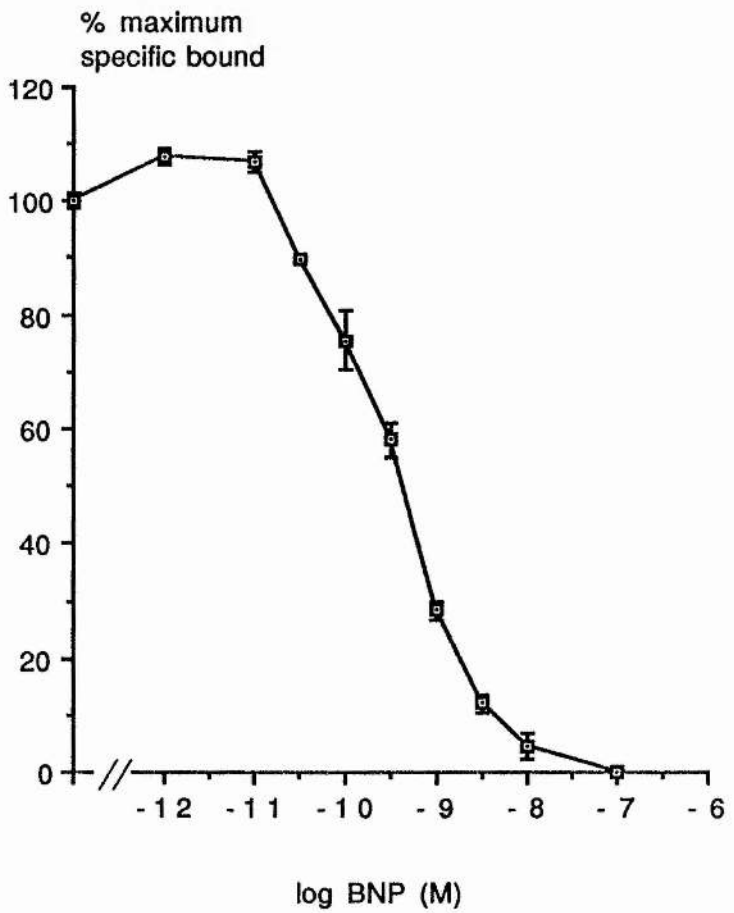
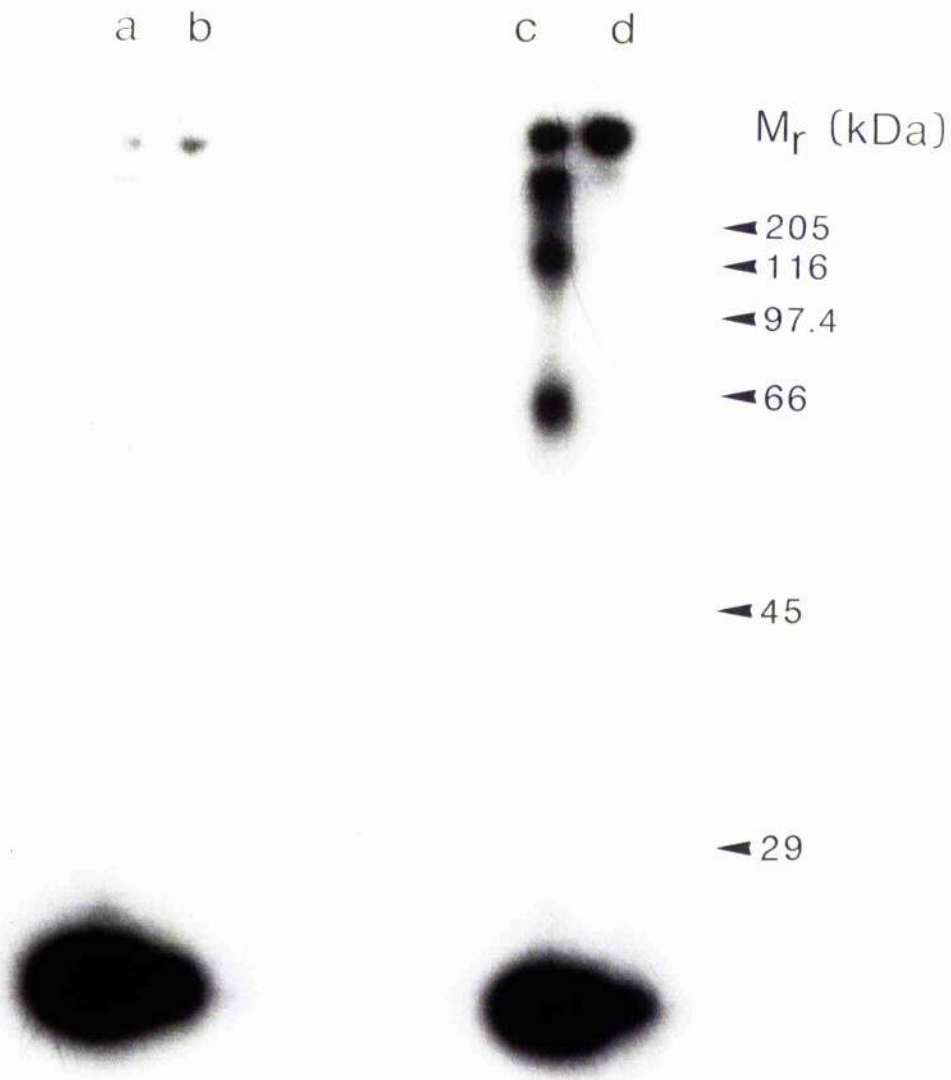


Figure 3.7

Crosslinking of [^{125}I]-ANP to bovine ventricular sarcolemmal membranes at room temperature in the presence or absence of β-mercaptoethanol.

[^{125}I]-ANP (50 pM) was incubated with BS membranes (700 μg) at room temperature, in the absence (lanes a and c) and in the presence (lanes b and d) of 0.1 μM ANP. Lanes a and b are in the presence and lanes c and d are in the absence of β-mercaptoethanol. Migration of the molecular weight standards is indicated.


a
b
c
d
M_r (kDa)
205
116
97.4
66
45
29

entering the SDS-PAGE gel. Urea (4 M) was included in the sample buffer at this time to aid the entry of this radiolabelled protein material into the gel. Urea was noted to increase the amounts of radiolabel entering the gels however the amounts of radiolabel present in wells with unlabelled ANP and [^{125}I]-ANP remained higher than in wells with [^{125}I]-ANP alone, (this result was constantly observed throughout the remaining crosslinking experiments). Crosslinking of [^{125}I]-ANP with BS membranes at 22 ^{o}C (see figs. 3.8, 3.9 a and 3.9 b) resulted in time-dependent increases in radioactivity for both of these receptor sites. In addition crosslinking with [^{125}I]-ANP and BS membranes at 4 ^{o}C for 6 hr was performed (see fig. 3.10). The displacement of [^{125}I]-ANP (50 pM) at 22 ^{o}C by unlabelled ANP was initially examined in the presence or the absence of β-mercaptoethanol (see fig 3.11). These results revealed that the radioactive signals from both of these receptor sites were lost if the samples were reduced with β-mercaptoethanol prior to electrophoresis. Two-dimensional SDS-PAGE was then performed, (see fig. 3.12) under non-reducing conditions (1st dimension) and then reducing conditions (2nd dimension). Two radiolabelled proteins of 60 kDa and 120 kDa were apparent after electrophoresis in the first dimension with no radiolabelled proteins greater than 5 kDa present after electrophoresis in a second dimension. The dissociation of [^{125}I]-ANP (50 pM) at 22 ^{o}C by various concentrations of unlabelled ANP (see fig 3.13) and des-ANP (see fig. 3.14 a and 3.14 b) was then investigated in crosslinking experiments under non-reducing conditions. The data from the scanned autoradiographs was expressed as a percentage of the maximum binding in the presence of 50 pM [^{125}I]-ANP alone (see fig. 3.15). The appropriate IC_{50} values were determined for each peptide and for each receptor protein. The IC_{50} values for ANP dissociation of [^{125}I]-ANP from the 120 kDa receptor protein and the 60 kDa receptor protein were 0.07 nM and 0.3 nM

Figure 3.8

Time course for the crosslinking of [^{125}I]-ANP to bovine ventricular sarcolemmal membranes at room temperature.

[^{125}I]-ANP (50 pM) was incubated with BS membranes (700 μg) at room temperature, for 180 min, (lanes (a) and (b)) 60 min (lanes (c) and (d)) and 10 min (lanes (e) and (f)). Lanes (a), (c) and (e) are in the presence of [^{125}I]-ANP and lanes (b), (d) and (f) are in the presence of [^{125}I]-ANP and 0.1 μM ANP. Migration of the molecular weight standards is indicated.

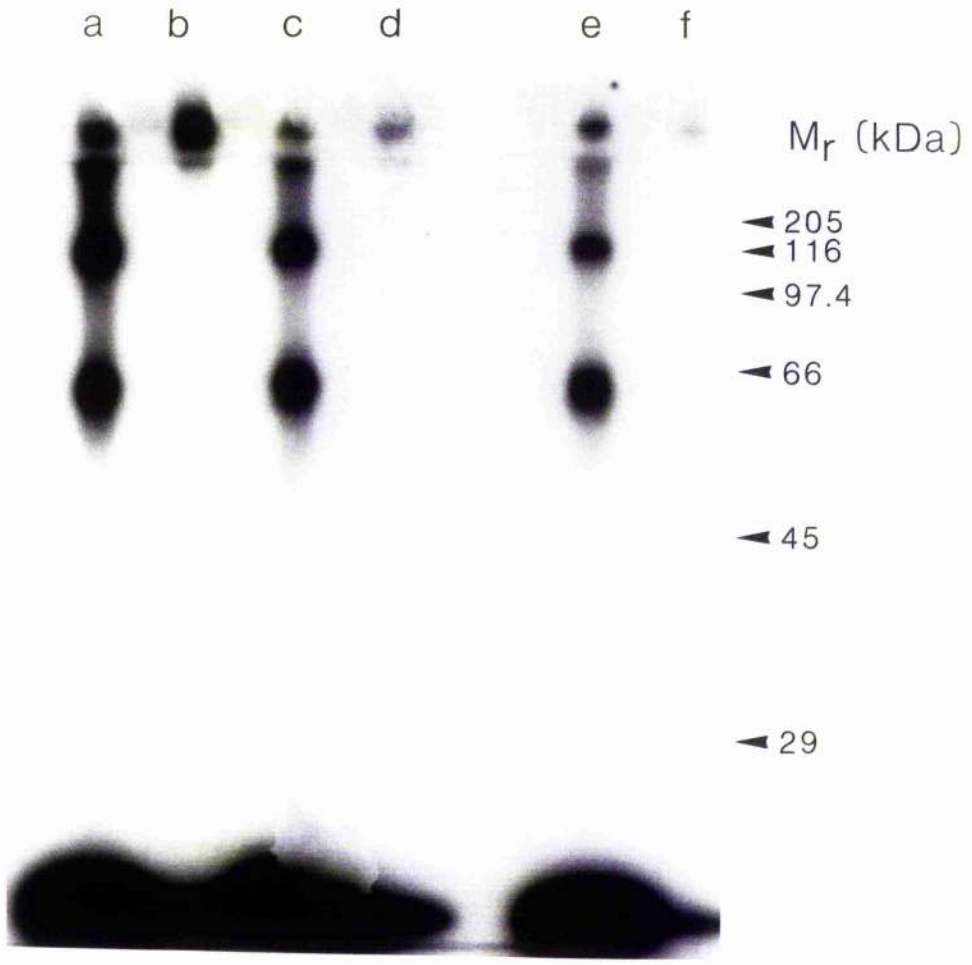

a
b
c
d
e
f
Mr (kDa)
205
116
97.4
66
45
29

Figure 3.9

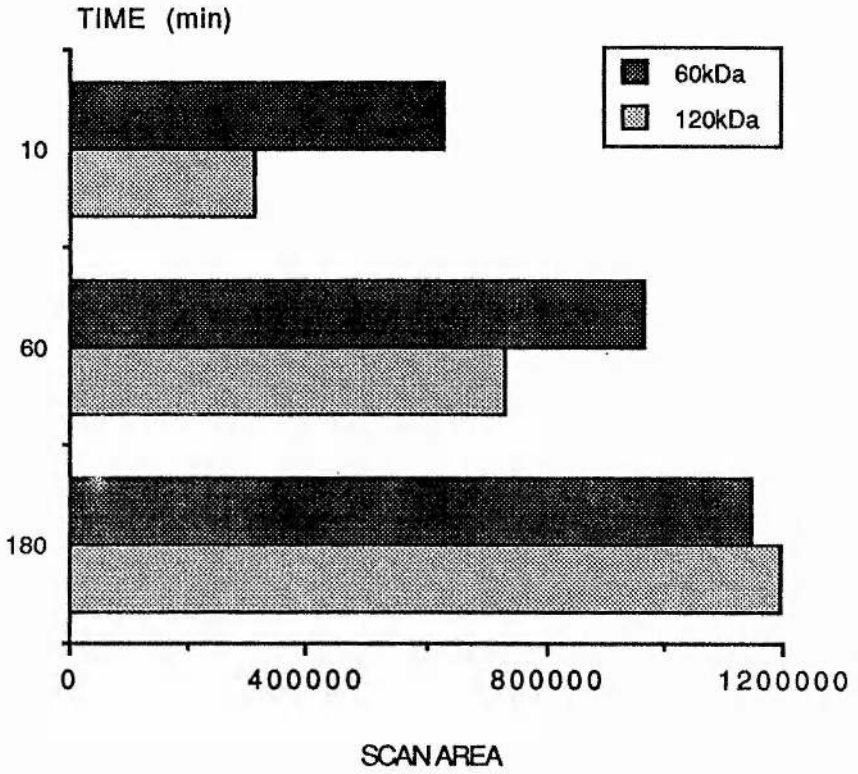
Time course for the crosslinking of [^{125}I]-ANP to bovine ventricular sarcolemmal membrane 60 kDa, 120 kDa receptor proteins and SDS-PAGE gel 'well' proteins.

[^{125}I]-ANP (50 pM) was incubated with BS membranes (700 μg) at room temperature, for 10 min, 60 min and 180 min.

(a) shows the increase in crosslinking of [^{125}I]-ANP to the 60 kDa and the 120 kDa receptor proteins with time.

(b) shows the increase in crosslinking of [^{125}I]-ANP in the well of the SDS-PAGE gel with time. The areas are the appropriate areas determined from the scanned autoradiograph of fig. 3.8.

a


TIME (min)
60kDa
120kDa
10
60
180
0
400000
800000
1200000
SCAN AREA

b

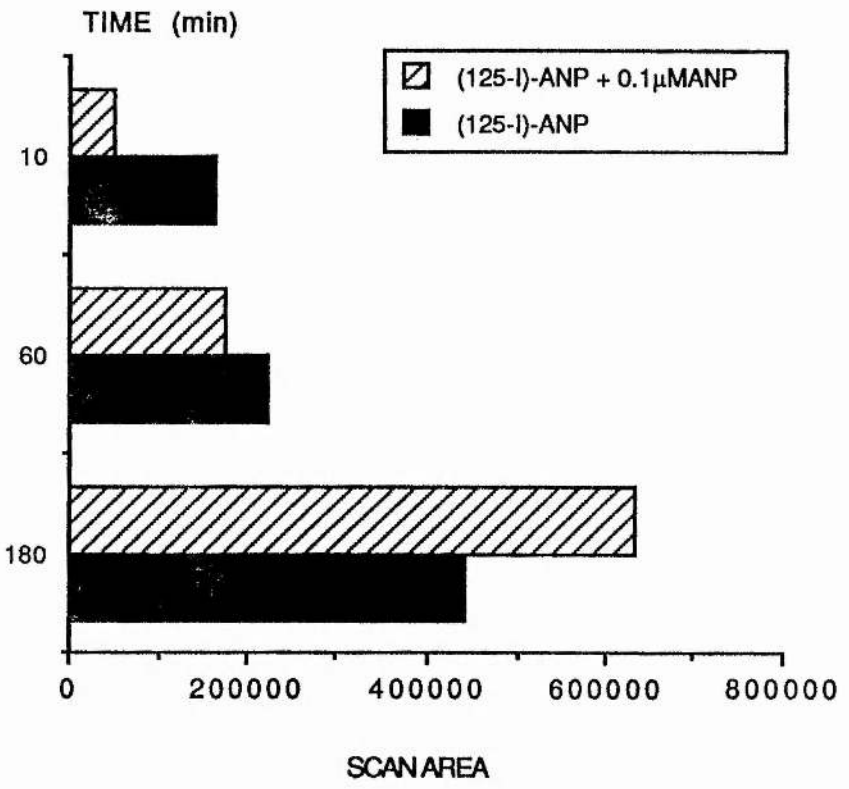

TIME (min)
(125-I)-ANP + 0.1μMANP
(125-I)-ANP
10
60
180
0
200000
400000
600000
800000
SCAN AREA

Figure 3.10

Crosslinking of [^{125}I]-ANP to bovine ventricular sarcolemmal Membranes for 6 hr at 4 ^{o}C in the absence or the presence of β-mercaptoethanol.

[^{125}I]-ANP (50 pM) was incubated with BS membranes (700 μg) at 4 ^{o}C for 6 h in the absence (lanes e, f, g and h) or in the presence (lanes a, b, c and d) of β-mercaptoethanol. lanes a, c, e, and g are in the presence [^{125}I]-ANP (50 pM) and 0.1μM ANP. Lanes b, d, f and h are in the presence of [^{125}I]-ANP (50 pM). Migration of the molecular weight standards is indicated.

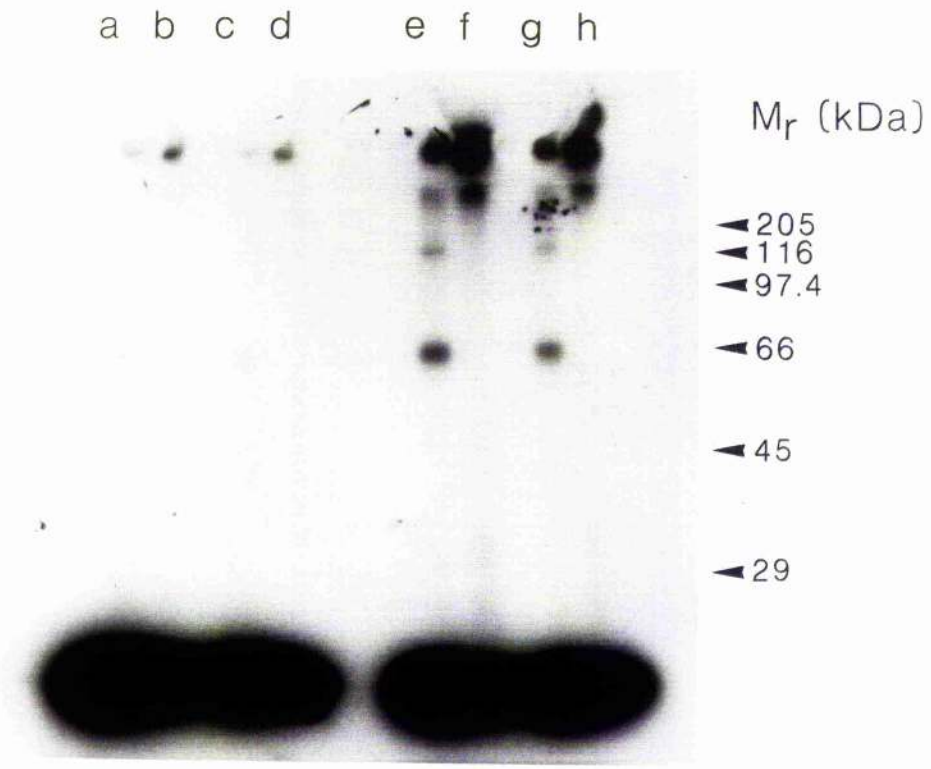

a b c d e f g h
Mr (kDa)
205
116
97.4
66
45
29

Figure 3.11

Displacement of crosslinked [^{125}I]-ANP to bovine ventricular sarcolemmal membranes at room temperature in the presence or absence of β-mercaptoethanol.

[^{125}I]-ANP (50 pM) was incubated with BS membranes (700 μg) at room temperature, in the absence (lanes g to l) and in the presence (lanes a and f) of β-mercaptoethanol. Lanes a and g are in the presence of [^{125}I]-ANP only. ANP at 10^{-7}M (lanes b and h), 10^{-8}M (lanes c and i), 10^{-9}M (lanes d and j), 10^{-10}M (lanes e and k) and 10^{-11}M (lanes f and l) is present in addition to [^{125}I]-ANP. Migration of the molecular weight standards is indicated.

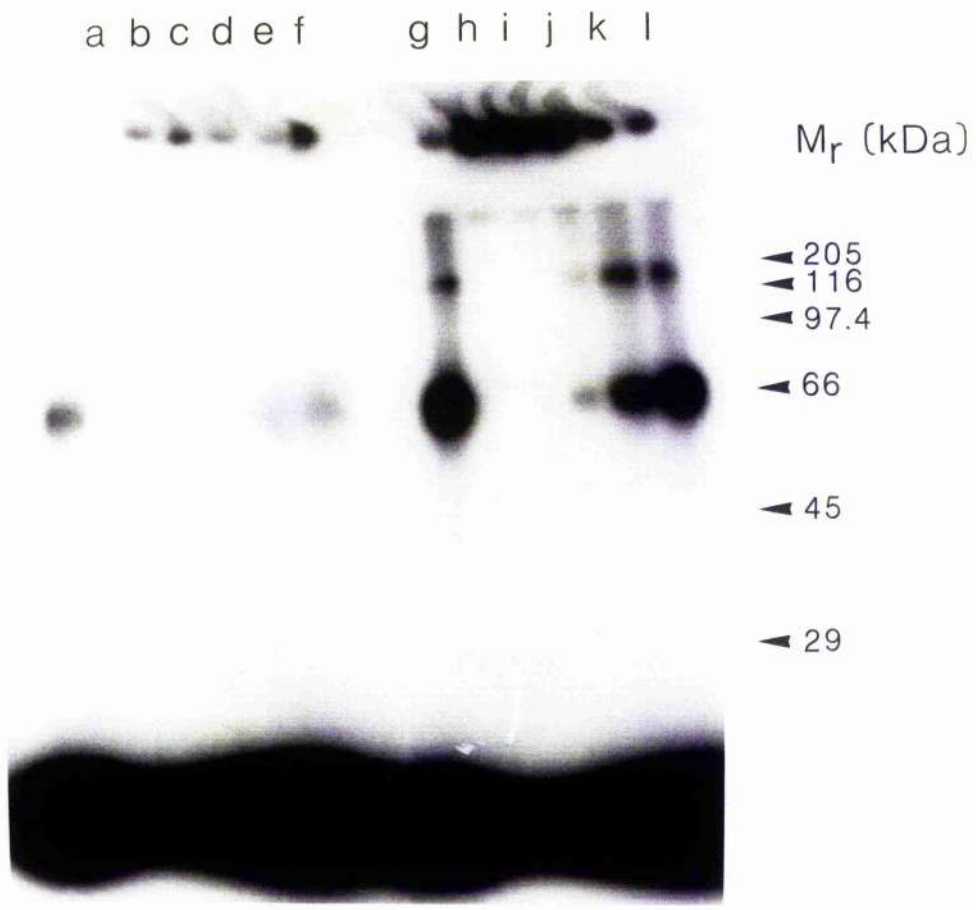

a b c d e f g h i j k l
Mr (kDa)
205
116
97.4
66
45
29

Figure 3.12

2-Dimensional SDS-PAGE of [^{125}I]-ANP crosslinked bovine ventricular sarcolemmal membranes

After [^{125}I]-ANP crosslinking, samples were subjected to two dimensional SDS-PAGE under non-reduced (1st. dimension) and then reduced (2nd. dimension) conditions. Migration of the molecular weight standards is indicated.

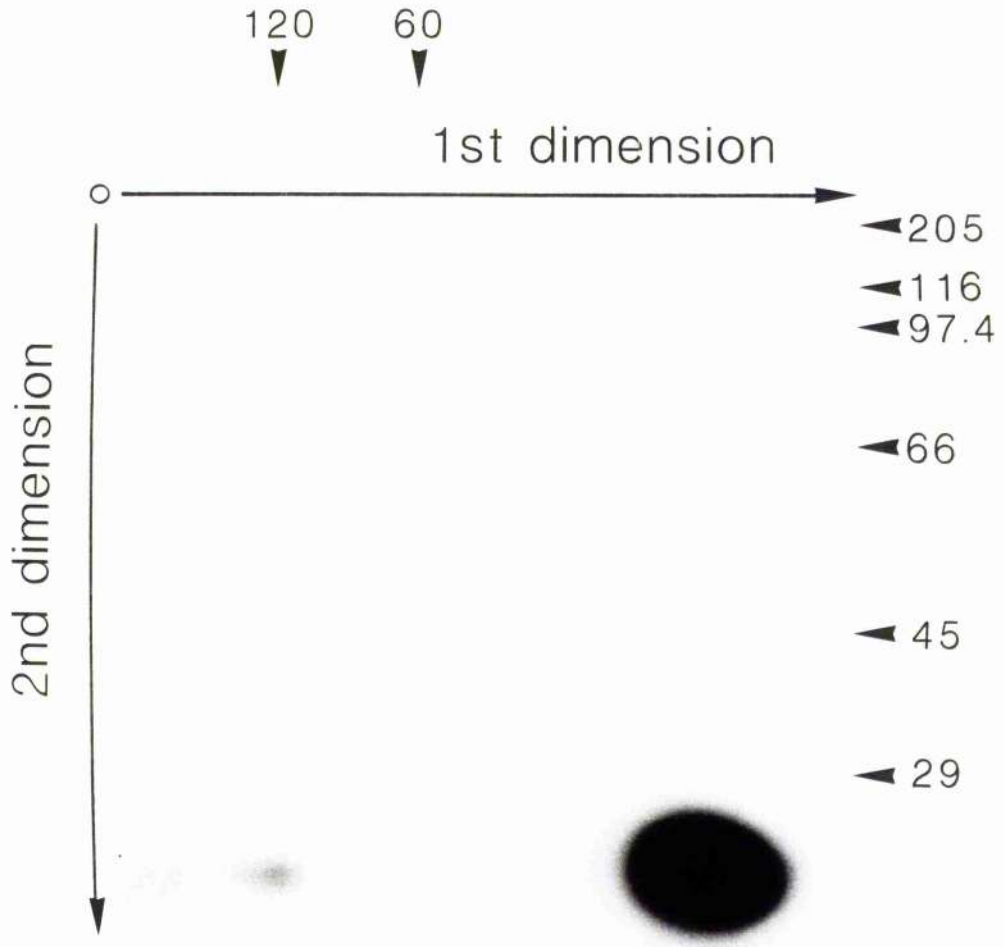

120
60
1st dimension
2nd dimension
205
116
97.4
66
45
29

Figure 3.13

Inhibition of [^{125}I]-ANP crosslinking to bovine ventricular sarcolemmal membranes at room temperature in the presence of ANP.

[^{125}I]-ANP (50 pM) was incubated with BS membranes (700 μg) at room temperature, in the absence (lanes a) and in the presence of 10^{-7}M (lanes b), 10^{-9}M (lanes c), 10^{-10}M (lanes d) 10^{-11}M (lanes e) and 10^{-12}M (lanes f) ANP. Migration of the molecular weight standards is indicated.

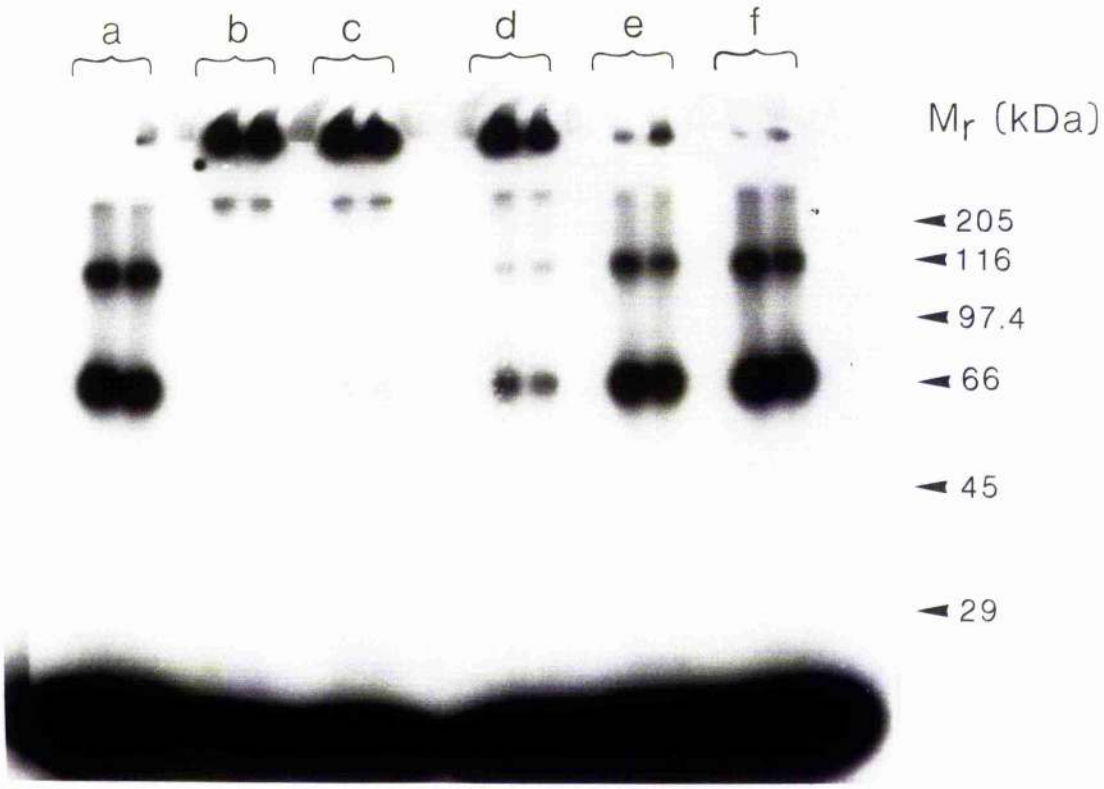

a
b
c
d
e
f
Mr (kDa)
205
116
97.4
66
45
29

Figure 3.14 a

Inhibition of [^{125}I]-ANP crosslinking to bovine ventricular sarcolemmal membranes at room temperature by des-ANP.

[^{125}I]-ANP (50 pM) was incubated with bovine cardiac sarcolemmal membranes (700 μg) at room temperature, the absence (lanes a) and in the presence of 10^{-6}M (lanes b), 10^{-7}M (lanes c), 10^{-8}M (lanes d) 10^{-9}M (lanes e) and 10^{-10}M (lanes f) des-ANP. Migration of the molecular weight standards is indicated.

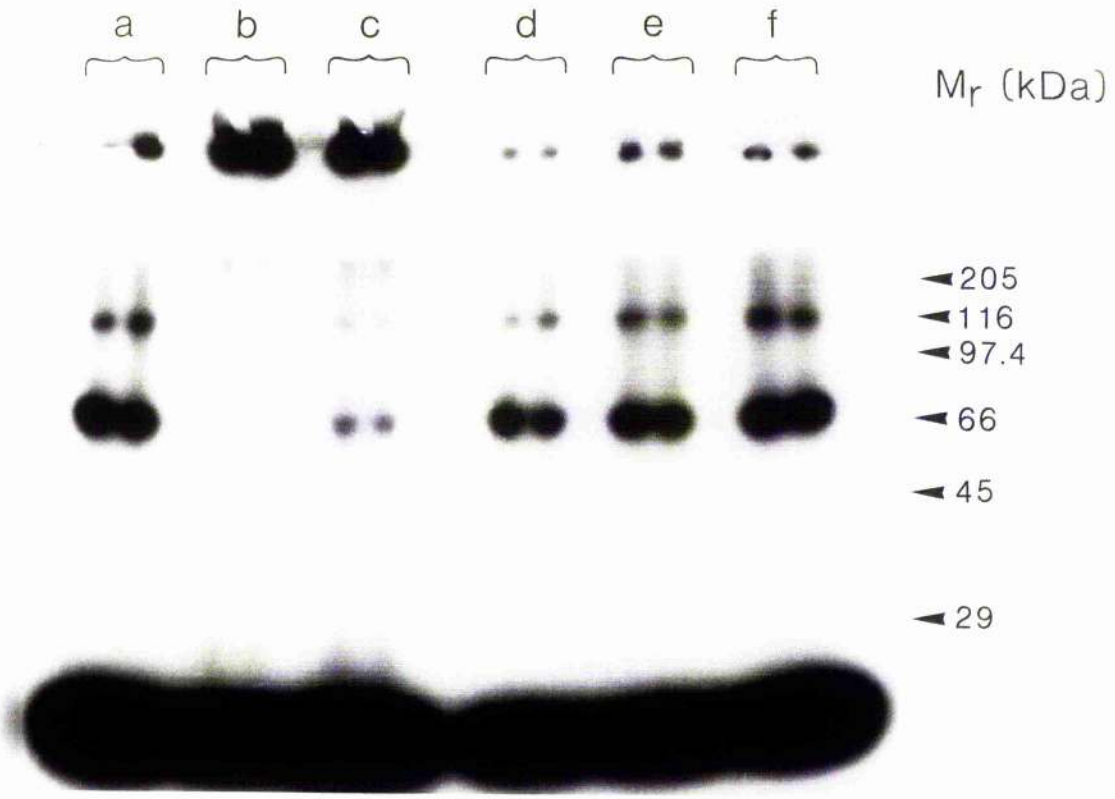

a
b
c
d
e
f
Mr (kDa)
205
116
97.4
66
45
29

Figure 3.14 b

Inhibition of [^{125}I]-ANP crosslinking to bovine ventricular sarcolemmal membranes at room temperature by des-ANP.

[^{125}I]-ANP (50 pM) was incubated with bovine cardiac sarcolemmal membranes (700 μg) at room temperature, in the absence (lanes a) and in the presence of 10^{-7}M (lanes b), 10^{-9}M (lanes c), 10^{-10}M (lanes d) 10^{-11}M (lanes e) and 10^{-12}M (lanes f) des-ANP. Migration of the molecular weight standards is indicated.

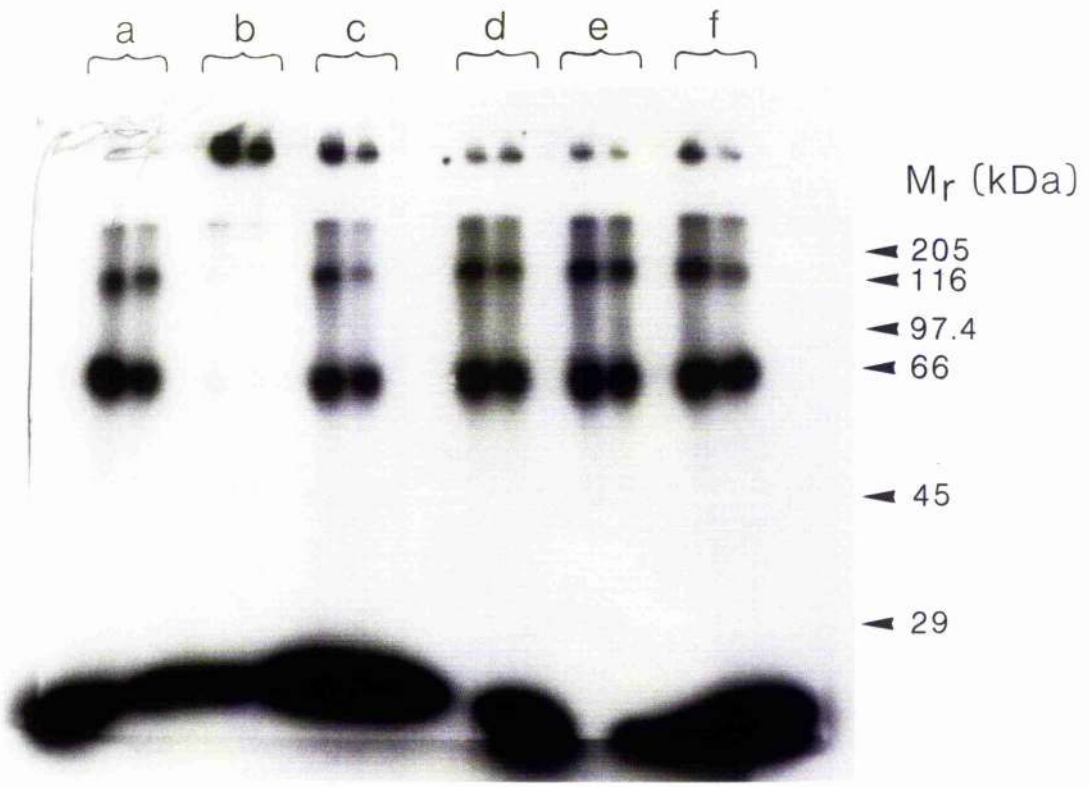
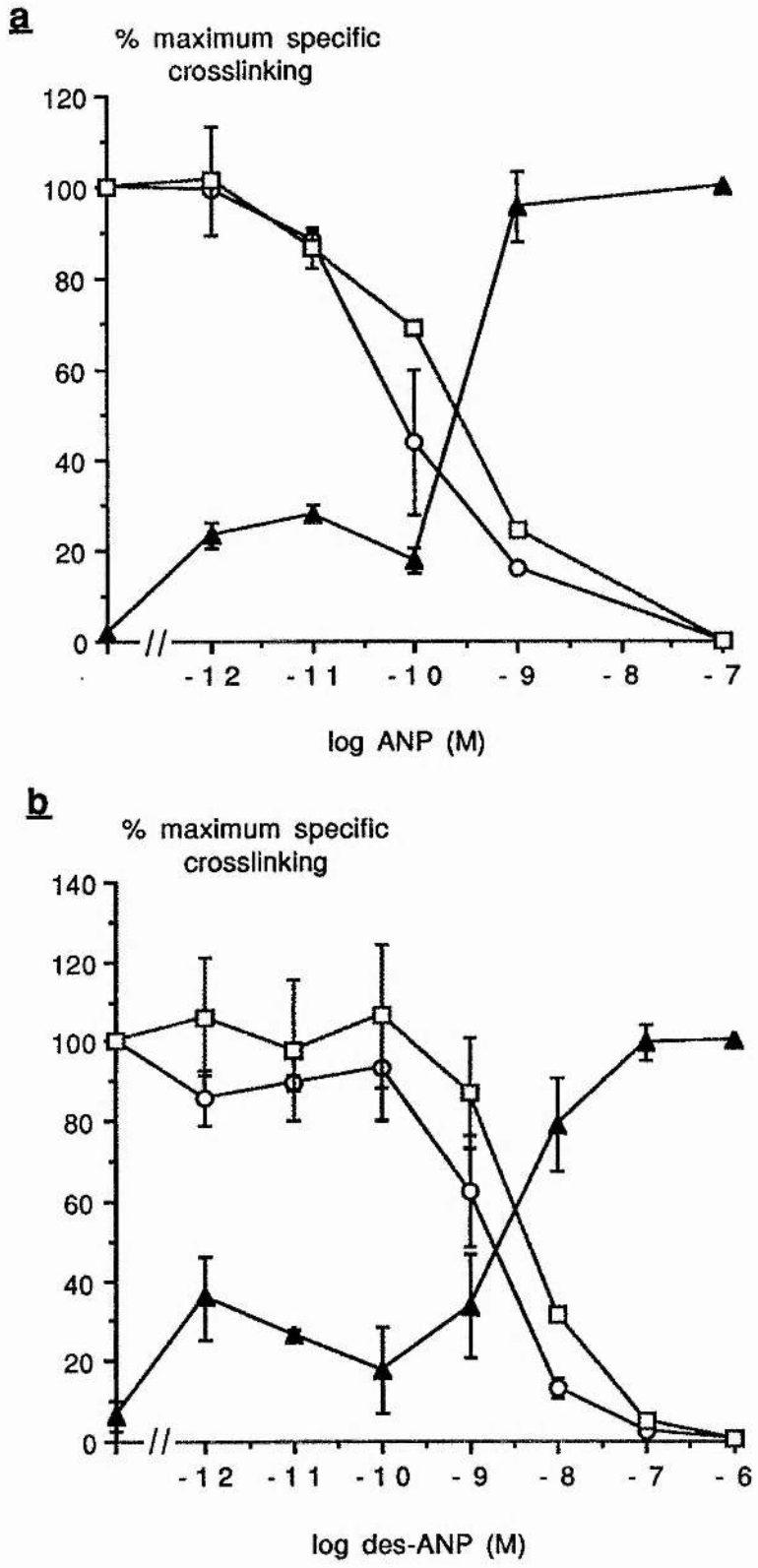

a
b
c
d
e
f
Mr (kDa)
205
116
97.4
66
45
29

Figure 3.15

Inhibition of [^{125}I]-ANP crosslinking to bovine ventricular sarcolemmal membranes at room temperature by ANP and des-ANP.

Dose response curves for the displacement of crosslinked [^{125}I]-ANP (50 pM) from BS membranes by increasing concentrations of ANP (a) and des-ANP (b).

(a) the areas are the appropriate areas determined from the scanned autoradiograph of fig. 3.13 and (b) the areas are the appropriate combined areas determined from the scanned autoradiographs of figs. 3.14 a and 3.14 b. Values are expressed as a % of the maximum crosslinked value from scanned autoradiographs of well (▲-▲), 60 kDa receptor protein (□-□) and 120 kDa receptor protein (O-O).


a
% maximum specific crosslinking
120
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
log ANP (M)
b
% maximum specific crosslinking
140
120
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
log des-ANP (M)

respectively. The IC_{50} values for des-ANP dissociation of [^{125}I]-ANP from the 120 kDa receptor protein and the 60 kDa receptor protein were 2 nM and 5 nM respectively. The EC_{50} values for the increases in radiolabel in the SDS-PAGE gel wells were 0.3 nM and 2 nM for ANP and des-ANP respectively.

3.4 Results of Guanylate Cyclase Experiments.

Incubation of BS membranes at 22 °C (see fig. 3.16) and 37 °C (see fig. 3.17) in the presence or the absence of 1 μM ANP resulted in time-dependent increases in guanylate cyclase activity as determined by radioimmunoassay for cGMP. The rate of cGMP production was constant for at least 20min, both in the presence and in the absence of ANP. The relative amount of cGMP production (nmol/μg protein) was greater at 37 °C than at 22 °C throughout the time courses. Subsequent experiments were performed for 20 min at 37 °C. The effects of ANP, des-ANP and Tyr^8-ANP on guanylate cyclase activity in BS membranes were examined. Manganese-dependent guanylate cyclase activity was measured in the presence of increasing concentrations of the peptides (see fig. 3.18). ANP, des-ANP and Tyr^8-ANP were all capable of stimulating guanylate cyclase activity by 46-100% of basal values. Atrial natriuretic peptide (ANP), des-ANP and Tyr^8-ANP stimulated guanylate cyclase activity from basal values of 60, 96 and 96 pmol/min/mg protein to values of 110, 146 and 191 pmol/min/mg protein respectively. The relative EC_{50} values for ANP, des-ANP and Tyr^8-ANP were 1 nM, 0.1-1 μM and 0.1 μM respectively.

3.5 Discussion

Analysis of the data from ligand binding experiments indicated the presence of a single high-affinity (K_d for [^{125}I]-ANP 43.6 ± 8.3 pM and K_d

Figure 3.16

Time course for the production of cGMP in bovine ventricular sarcolemmal membranes at room temperature.

A representative time course for guanylate cyclase activity in the absence (□-□) and in the presence (■-■) of 1 μM ANP. The assay was conducted at room temperature as described in the Materials and Methods. Each point represents the mean ± S.D. from one experiment with triplicate determinations of guanylate cyclase activity, followed by duplicate determinations of cGMP.

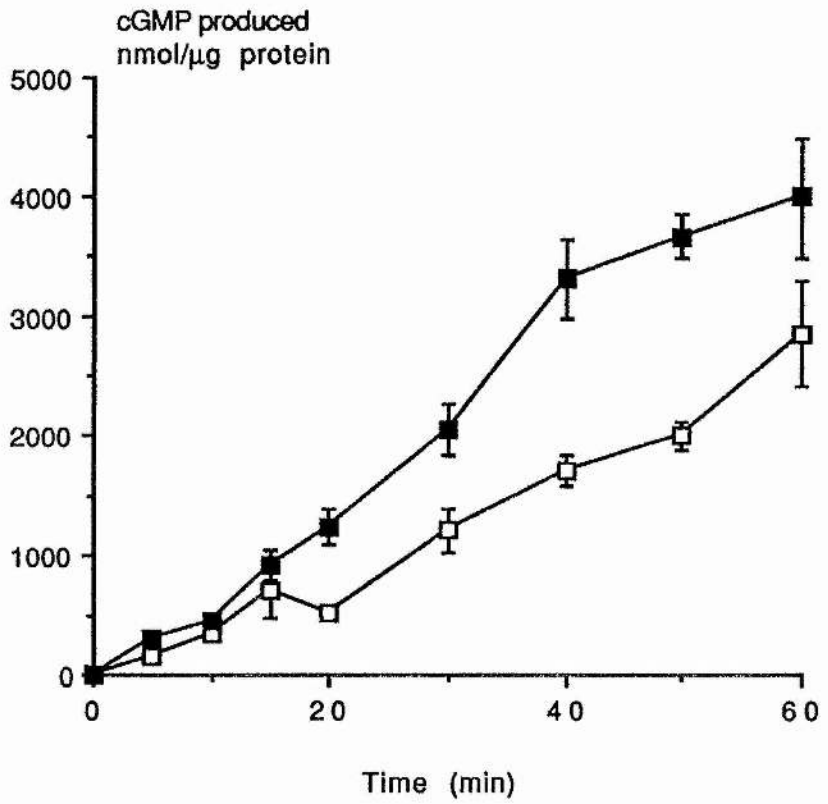


Figure 3.17

Time Course for the production of cGMP in bovine ventricular sarcolemmal membranes at 37 °C.

A representative time course for guanylate cyclase activity in the absence (□-□) and in the presence (■-■) of 1 μM ANP. The assay was conducted at 37 °C as described in the Materials and Methods. Each point represents the mean ± S.D. from one experiment with triplicate determinations of guanylate cyclase activity, followed by duplicate determinations of cGMP.

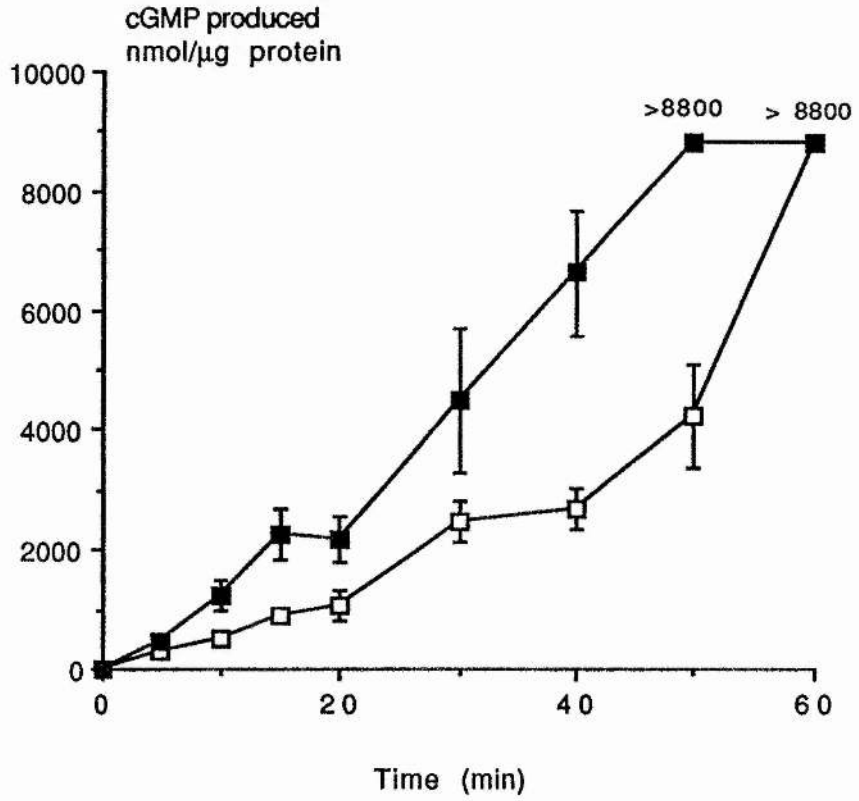
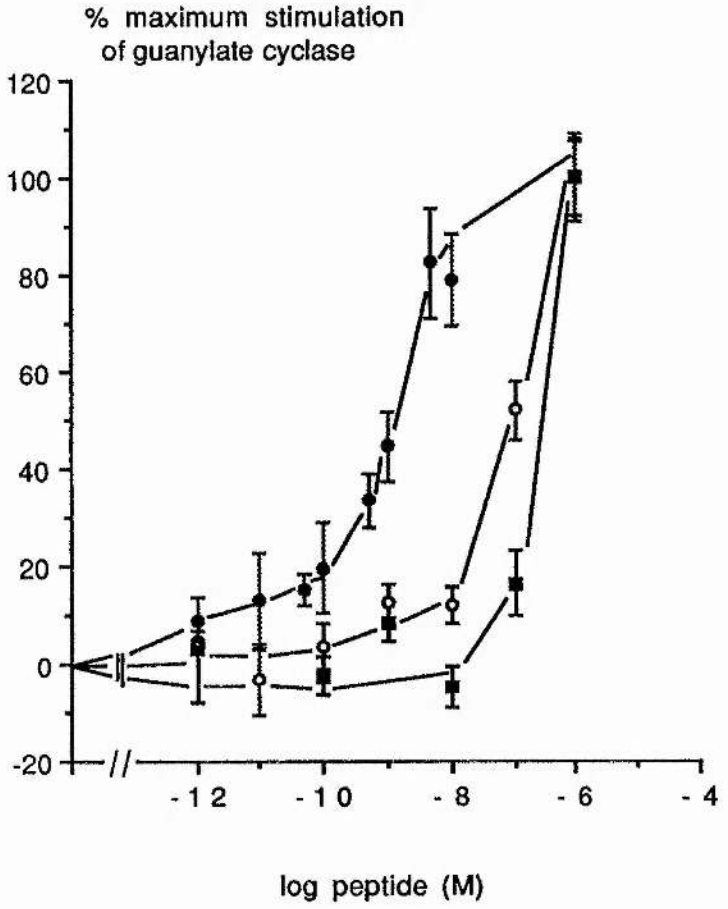


Figure 3.18

Stimulation of guanylate cyclase activity in BS membranes by ANP, des-ANP and Tyr^8-ANP.

BS membranes (10 μg/20 μl) were incubated at 37 °C for 20 min with increasing concentrations of ANP (●-●), des-ANP (■-■) and Tyr^8-ANP (○-○). Points for des-ANP and Tyr^8-ANP represent the mean ± S.E.M. of six individual measurements taken from one experiments and points for ANP represent the mean ± S.E.M. of twelve individual measurements taken from two separate experiments.



for ANP 65 ± 27.9 pM) binding site in sarcolemmal membranes isolated from the bovine ventricular myocardium. The ANP receptor population was found to bind the ANP analogues des-ANP and Tyr^8-ANP with a 7000 and 7-fold lower affinity respectively than ANP. The calculated K_d's for ANP, ANP in TX-100 treated membranes, des-ANP and Tyr^8-ANP (determined from IC_{50} values) were 65 pM, 18.6 pM, 486 nM and 466 pM respectively. The ANP-C receptor has been characterised in other tissues and has been shown to possess equal binding affinity to ANP as that of various ANP analogues, including des-ANP (Maack *et al.* 1987; Inagami *et al.* 1988 and Lewicki *et al* 1988). The binding results therefore indicate that the ANP-B receptor is the predominant receptor population in BS membranes. The binding of BNP to BS membranes with an affinity similar to that of ANP indicates that BNP is perhaps binding to the same receptor site as ANP as suggested in other tissues (Hirata *et al.* 1988; Song *et al.* 1988; Gelfand *et al.* 1991), however further crosslinking and guanylate cyclase analysis with BNP is required to support this observation. The presence of ANP-B receptors in BS membranes is further indicated by the fact that guanylate cyclase activity was stimulated up to 2-fold by ANP. Surprisingly, des-ANP and Tyr^8-ANP were both able to stimulate guanylate cyclase activity up to 1.5 to 2-fold in BS membranes however the concentrations required were in excess of 100 nM. The guanylate cyclase results obtained with Tyr^8-ANP are in agreement with that of Budzik *et al.* (1987) who observed that although low concentrations of Tyr^8-ANP possessed potent vasorelaxant properties the peptide lacked the ability to stimulate cGMP production, at these low concentrations. A poor correlation between ANP ligand binding and the subsequent stimulation of guanylate cyclase was noted in BS membranes. The K_d values obtained for ANP, des-ANP and Tyr^8-ANP binding were found to be 10-100 fold lower than the EC_{50} values required for activation of

guanylate cyclase. Schenk *et al.* (1985a) in bovine aortic smooth muscle and endothelial cells and Pandey *et al.* (1988) in rat aortic smooth muscle cells and kidney tubular epithelium cells have previously reported similar discrepancies. The lack of correlation between these two values has previously been explained on the basis of ANP receptor heterogeneity and the predominance of ANP-C receptors in the tissues or cells examined. However the second senario cannot be true of BS membranes since the competitive displacement binding analysis with des-ANP indicated a predominance, greater than 90% of ANP-B receptors. It is possible that the relationship between the activation of guanylate cyclase activity and ANP ligand binding is more complex than was at first postulated. This has been recently indicated in ANP receptor cloning studies, where a family of inter-related ANP receptor populations has been reported, (see section 1.2.5.1).

Crosslinking studies in BS membranes clearly indicated the presence of two receptor proteins, one of 60 kDa and one of 120 kDa in size, initially thought to be the ANP-C and ANP-B receptor populations respectively. The radioactive signals from both of these receptor sites were lost if the samples were reduced with β-mercaptoethanol prior to electrophoresis. Pandey *et al.* (1987a; 1987b; 1988) and Takayanagi *et al.* (1987a) have reported the loss of all or nearly all of the radioactive signal from the 120-140 kDa receptor but not from the 60-70 kDa receptor under sample reducing conditions. This result has been interpreted as indicative of the existence of a third ANP receptor, which possibly exists as a dimer of the ANP-C receptor and is reduced to half its molecular weight after treatment with β-mercaptoethanol or DTT. The unusual results found with BS membranes may indicate an alternative explanation. It is possible that the binding of [^{125}I]-ANP to both of these membrane receptors is associated

with the hydrolysis of one or more of the peptide bonds within the 17 amino acid loop structure of the [^{125}I]-ANP molecule. Under non-reducing conditions this would allow the ^{125}I-radiolabeled tyrosine at the C-terminal of the [^{125}I]-ANP molecule to remain attached to the N-terminal of the [^{125}I]-ANP molecule via the disulphide bond and thus remain attached to the receptor itself. In the presence of β-mercaptoethanol the disulphide bond connection between the C-terminal and the N-terminal of the hydrolysed [^{125}I]-ANP molecule would be reduced and the radiolabelled C-terminal of the [^{125}I]-ANP molecule would be lost from the ANP-receptor unit. These results with BS membranes therefore indicate an alternative explanation for the loss of high molecular weight receptor radiolabel found in crosslinking studies in other tissues. Crosslinking experiments investigating the competition of [^{125}I]-ANP with various concentrations of unlabelled ANP indicate that [^{125}I]-ANP binding is lost from both of the identified receptor proteins in a concentration dependent manner and both possess similar IC_{50} values. Surprisingly, crosslinking experiments investigating the competition of [^{125}I]-ANP with the ANP analogue, des-ANP also indicate that [^{125}I]-ANP binding is lost from both receptor proteins with similar IC_{50} values and with values that were only 16-30 fold higher than those of ANP. This result was unexpected. It was thought that des-ANP would be able to compete with [^{125}I]-ANP binding at the 60 kDa 'ANP-C' receptor and would be unable to compete with [^{125}I]-ANP binding at the 120 kDa 'ANP-B' receptor in BS membrane preparations. It has to be remembered that crosslinking efficiency is extremely low, as is evident from the intense amounts of radiolabel present at the bottom of the SDS-PAGE gel autoradiographs. This [^{125}I]-ANP radiolabel binding is specific since lanes with [^{125}I]-ANP and unlabelled ANP show less radioactivity running at the dye front than lanes with [^{125}I]-ANP alone; however the DSS seems to

be unable to crosslink this specific [^{125}I]-ANP radiolabel to its respective receptor protein. The precise efficiency of DSS in crosslinking [^{125}I]-ANP to each of these two individual receptor sites under SDS-PAGE conditions remains to be fully determined. These results can possibly be interpreted as follows; the crosslinker DSS in BS membrane preparations is unable to crosslink [^{125}I]-ANP to the 120 kDa 'ANP-B' receptor and the 120 kDa receptor protein which was identified in these crosslinking experiments may be a dimer of the 60 kDa 'ANP-C' receptor. Therefore des-ANP in the crosslinking experiments was able to competitively displace [^{125}I]-ANP from a 60 kDa 'ANP-C' receptor and a 120 kDa 'ANP-C' receptor dimer. This hypothesis may also explain why in the crosslinking experiments the 120 kDa 'ANP-B' receptor is not found to predominate over the 60 kDa 'ANP-C' receptor protein in BS membranes as would be expected from the results of the radio-receptor assays. The presence of the ANP-B receptor with associated guanylate cyclase activity is therefore only identified in radioreceptor binding and guanylate cyclase experiments where this receptor population is indicated as being in the majority.

The experiments described demonstrate the presence of [^{125}I]-ANP receptors in purified bovine ventricular sarcolemmal preparations and ANP-stimulated guanylate cyclase activity in the same preparations. Results also provide biochemical evidence for the presence of at least three different ANP populations of receptors in the bovine ventricle. Specific receptors for ANP were identified as having similar molecular weights and some similar biochemical characteristics as ANP receptors identified in other tissues and cells, (see section 1.2.2). In addition, results extend earlier experimental studies from this laboratory where the presence of two affinity classes of ANP receptors were demonstrated in purified rat cardiac sarcolemmal preparations, (Rugg *et al.* 1989); one

receptor possessing ANP-stimulated guanylate cyclase activity. ANP-stimulation of intracellular cGMP levels in isolated rat and rabbit ventricular myocytes (Aiton & Cramb 1985; Cramb *et al.* 1987) has also been demonstrated in this laboratory. The concentration of ANP required to produce half maximal stimulation of cGMP in the bovine sarcolemma, is similar to the concentration required in the rat sarcolemma, however this concentration is approximately 10-fold less than that required for half maximal elevation of cGMP in intact rat or rabbit myocytes. Lang *et al.* (1985) and Gutkwoska *et al.* (1984) found that plasma ANP concentrations were 2-3 fold less than the half maximal value required for stimulation of guanylate cyclase activity. This discrepancy is most likely due to the rapid degradation of ANP which occurs when ANP is incubated with ventricular myocytes, (Cramb *et al.* 1987). It should be noted that James *et al.* (1990) observed no ANP binding sites on either atrial or ventricular myocytes in rat and guinea-pig cultures. This group suggested that the conflicting evidence, with regards to the presence/absence of ANP binding sites in cardiac muscle was perhaps a result of problems in preparing completely homogenous suspensions of cells from the heart and that the ANP receptors previously identified were in fact associated with non-muscle cells. Morkin & Ashford (1968) showed that myocytes only constitute approximately 27% of the myocardial cells with connective tissue cells (35%) and blood vessel endothelial cells (38%) together constituting 73%. Leitman *et al.* (1986) using both radio-receptor assays and crosslinking experiments showed in endothelial cells, that greater than 90% of the total ANP receptors were ANP-C receptors, whereas radio-receptor assays in bovine sarcolemmal membrane preparations, indicated that greater than 90% of the total ANP receptor density were ANP-B receptors (McCartney *et al.* 1990). These results indicate that it is unlikely that the ANP receptors identified in bovine cardiac muscle preprarations

are associated with non-muscle endothelial cells. Alternatively, James *et al.* (1990) suggested that the apparent absence of ANP binding sites on rat and guinea-pig cultured myocytes may have been a result of the culture conditions. Patterson and Chun (1974) and Mudge (1981) have previously shown that growth medium conditioned by non-neuronal cells can alter the phenotype of some neurones in culture. Another possibility, proposed by Rugg *et al.* (1988) is that ANP is rapidly degraded by a soluble, heat labile peptidase isolated from ventricular myocytes. The apparent absence of ANP binding sites on rat and guinea-pig cultured myocytes may then be a result of rapid proteolytic degradation of ANP and a subsequent lack of ANP-specific receptor binding. The presence of ANP binding sites in bovine sarcolemmal membrane preparations may then be a result of the use of a 'cocktail' of protease inhibitors which ultimately prevents rapid proteolytic degredation of ANP and allows for ANP-specific receptor binding.

There is controversy concerning the vasorelaxant actions of ANP and the role of cGMP as the second messenger mediating these actions, (see section 1.1.7.3). Deth *et al.* (1982) and Currie *et al.* (1983) have shown direct evidence of a rat atrial extract causing relaxation of aortic strips. The results presented indicate that in bovine ventricular sarcolemmal preparations, ANP stimulates cGMP production. How the resultant stimulation of cGMP by ANP acts in the heart is not as yet fully known. However, work reported by George *et al.* (1970), George *et al.* (1973), Endoh (1979) and Lincoln & Keely, (1980) has shown a correlation between increased intracellular cGMP levels and force of contraction of ventricular muscle. Watanabe & Besch, (1975) have shown that dibutyryl cGMP antagonises the positive contractile effect of isoprenaline. Therefore cGMP antagonises agonist-induced positive contractile effects, with little

or no effect on the resting tension, (Endoh, 1979; Watanabe & Besch, 1975). Rugg (1989) hypothesised that ANP-stimulated cGMP production may also result in a negative contractile effect on ventricular muscle. Contrary to this Bohm *et al.* (1988) have shown that ANP has no contractile action in the rat and human heart.

Alternatively, ANP receptors present on ventricular muscle may form part of a negative feedback mechanism. ANP has been found to be present in ventricular muscle and this raises the possibility that the release of ANP from granules in the atria may modulate ventricular ANP gene expression and hence the release of ANP from the ventricles.

In conclusion, the results presented provide biochemical evidence for a direct action of ANP on bovine ventricular muscle. In addition, they provide evidence of the subtype, molecular weight and density of ANP receptors in the bovine ventricle. There is no doubt that the presence of these ANP specific receptors in the bovine ventricle present a complex insight as to the actions of ANP in this tissue. Further studies are required on the molecular biology of the ANP system to determine the precise nature of the ANP receptors present in the ventricle and to determine the physiological role of ANP in the heart.

3.6 Summary

The above radioreceptor binding data indicates in bovine ventricular sarcolemmal membranes the presence of only one ANP receptor population/class/subtype, the ANP-B receptor. In addition there is poor correlation between the calculated dissociation constant for ANP and the half maximum concentration required for the stimulation of guanylate cyclase activity. Crosslinking studies with DSS however, do not indicate

doubt that the presence of these ANP specific receptors in the bovine ventricle present a complex insight as to the actions of ANP in this tissue

3.7 Future Perspectives

The controversy which exists over the presence or absence of ANP receptors in the ventricular sarcolemma is obviously the most vital issue and awaits further investigation. Future experimental studies lie in the isolation of ANP receptors from purified myocyte preparations. This work should involve refining receptor binding assay techniques and the use of various crosslinkers under various experimental conditions. Further studies are also required on the molecular biology of the ANP system to determine the precise nature of the ANP receptors present in the ventricle and to determine the physiological role of ANP in the heart.

CHAPTER 4

RESULTS AND DISCUSSION OF PARTIALLY PURIFIED RAT LIVER PLASMA MEMBRANE EXPERIMENTS

4.1 Introduction

As part of an on-going investigation in this laboratory to assess the effects of dietary salt regime on plasma [ANP], ANP-specific receptor binding kinetics and ANP-specific receptor gene expression in the Dahl hypertension resistant (Dahl-R) and Dahl hypertension sensitive (Dahl-S) rat, partially purified liver plasma membrane homogenates (prepared as described in the Materials and Methods) from male Dahl-R and Dahl-S rats were used to determine ANP receptor population and density. The effects of resistance and sensitivity of the two rat strains to the development of hypertension, in conjunction with a variation in dietary salt regime on the density (B_{max}), population (ANP-C or ANP-B receptors) and affinity (K_d) of ANP specific receptors were assessed. At 5 weeks of age the Dahl-rats were all allowed free access to food of a normal salt diet (0.8% NaCl) and then after 10 days the rats were split into groups which continued on a normal diet (0.8% NaCl) and groups which were given access to a high-salt diet (8% NaCl). The rats were killed after a further 5 weeks and their tissues kept at - 90 ^{o}C until required. Therefore four groups of rats were examined; (a) Dahl-R rats on a 0.8% NaCl diet (b), Dahl-R an 8% NaCl diet (c), Dahl-S rats on a 0.8% NaCl diet and (d) Dahl-S rats on an 8% NaCl diet. For comparitive studies partially purified liver plasma membranes were also isolated from male Wistar rat and the density and affinity of ANP receptors were assessed.

4.2 Blood Pressure determinations.

The blood pressures of a subset of male Dahl-R and Dahl-S rats from each group were determined as described in the Materials and Methods (see fig. 4.1, table 4.1 and table 4.2). The results obtained indicate that the systolic blood pressure of the resistant rats on an 8% NaCl diet, (165 ± 11.4 mmHg) (group b) was significantly higher than that of the resistant rats on a 0.8% NaCl diet (140.8 ± 18.7 mmHg) (group a). A similar significant difference was also noted between the two groups of sensitive animals, (group d compared to group c) with significantly higher values of systolic (214.6 ± 17.6 mmHg compared to 181.5 ± 13.4 mmHg) and mean blood pressures (154.2 ± 10 mmHg compared to 133 ± 16.9 mmHg) . When the resistant and the sensitive animals on a 0.8% NaCl diet are compared, (group a and group c) there are significantly higher systolic, mean and diastolic blood pressures in the sensitive group. A similar significant response is noted when the resistant and the sensitive animals on an 8% NaCl diet, (group b and group d) are compared with significantly higher systolic, mean and diastolic blood pressures in the sensitive group.

4.3 Receptor-Binding Experiments

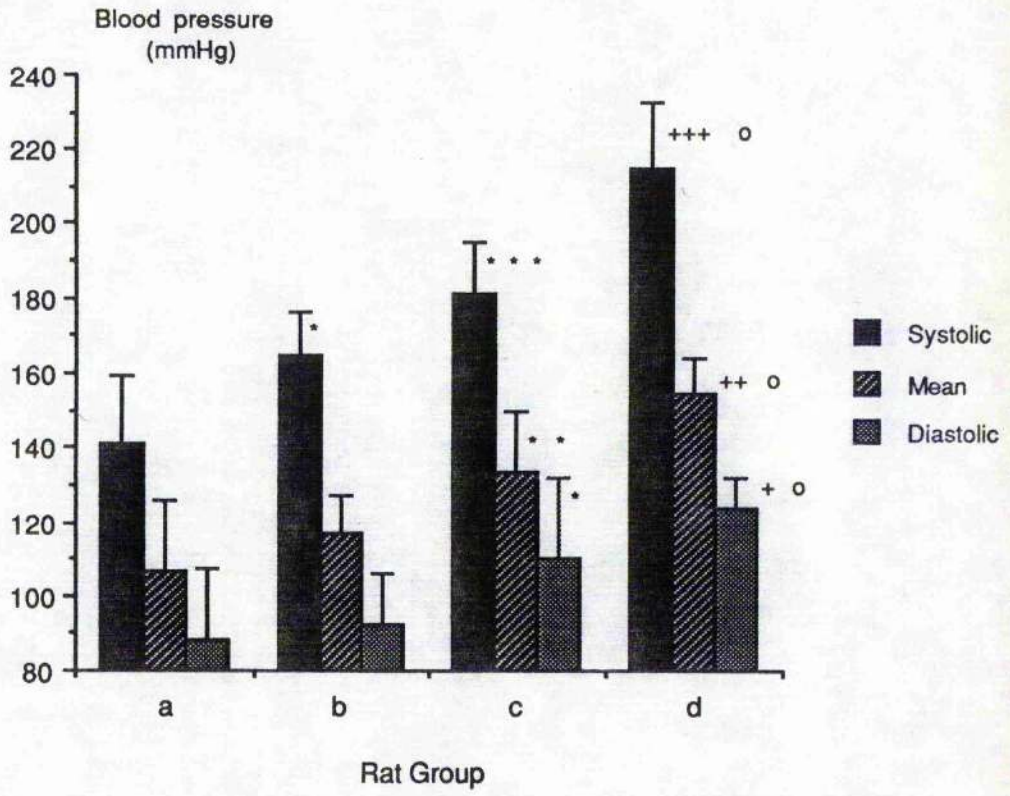
4.3.1 [^{125}I]-ANP receptor binding to Dahl rat liver plasma membranes.

Competitive displacement of 50 pM [^{125}I]-ANP from Dahl rat liver plasma membranes by ANP was carried out over a 1 h incubation period at room temperature. The cocktail of inhibitors present in the incubation buffer, as described in the Materials and Methods contained 0.1 μM phosphoramidon, (a specific inhibitor of endopeptidase 24.11) and 0.1 mM PMSF (a general serine protease inhibitor). Individual determinations were made using three separate liver membrane preparations for each of groups (a), (b), (c) and (d) (see fig. 4.2). Results are expressed as [^{125}I]-ANP bound (fmol/mg protein) and the results of the three separate

Figure 4.1

Systolic, Mean and Diastolic blood pressure measurements for Dahl-R and Dahl-S rats on different salt diets.

The results shown, are for (a) Dahl-R rats on a 0.8% NaCl diet, (b) Dahl-R rats on an 8% NaCl diet, (c) Dahl-S rats on a 0.8% NaCl diet and (d) Dahl-S rats on an 8% NaCl diet. Data for (a) is the mean of 8 "assessments" taken from 3 rats on 3 occasions, data for (b) is the mean of 12 "assessments" taken from 3 rats on 4 occasions, data for (c) is the mean 9 "assessments" taken from 3 rats on 3 occasions and data for (d) is the mean of 12 "assessments" taken from 3 rats on 4 occasions. Each blood pressure "assessment" is the mean of 6 to 12 individual readings taken in a series of consecutive measurements from one rat.



* significantly different from Dahl-R rats on an 0.8% NaCl diet ($p \leq 0.05$)

** significantly different from Dahl-R rats on a 0.8% NaCl diet ($p \leq 0.01$)

*** significantly different from Dahl-R rats on a 0.8% NaCl diet ($p \leq 0.001$)

+ significantly different from Dahl-S rats on an 0.8% NaCl diet ($p \leq 0.05$)

++ significantly different from Dahl-S rats on an 0.8% NaCl diet ($p \leq 0.01$)

+++ significantly different from Dahl-S rats on an 0.8% NaCl diet ($p \leq 0.001$)

o significantly different from Dahl-R rats on an 8% NaCl diet ($p \leq 0.001$)

Table 4.1

Mean ± SD for Systolic, Mean and Diastolic blood pressures (mmHg) of male Dahl-R and Dahl-S rats.

	Mean ± SD		
Rat Groups	Systolic	Mean	Diastolic
a	140.8 ± 18.7	106.5 ± 19	88.1 ± 19.1
b	165 ± 11.4 *	117.2 ± 10.3	92.6 ± 13.6
c	181.5 ± 13.4 ***	133 ± 16.9 **	109.8 ± 22.1*
d	214.6 ± 17.6 +++ o	154.2 ± 10 ++ o	123.9 ± 8 + o

* significantly different from Dahl-R rats on an 0.8% NaCl diet ($p \leq 0.05$)

** significantly different from Dahl-R rats on a 0.8% NaCl diet ($p \leq 0.01$)

*** significantly different from Dahl-R rats on a 0.8% NaCl diet ($p \leq 0.001$)

+ significantly different from Dahl-S rats on an 0.8% NaCl diet ($p \leq 0.05$)

++ significantly different from Dahl-S rats on an 0.8% NaCl diet ($p \leq 0.01$)

+++ significantly different from Dahl-S rats on an 0.8% NaCl diet ($p \leq 0.001$)

o significantly different from Dahl-R rats on an 8% NaCl diet ($p \leq 0.001$)

Table 4.2

***t* values for Systolic, Mean and Diastolic blood pressures of male Dahl-R and Dahl-S rats after dietary salt regime.**

	t value		
Rat Groups	Systolic	Mean	Diastolic
a 'v' b	3.61 *	1.635	0.617
c 'v' d	4.711 ***	3.603 **	2.501 *
a 'v' c	5.45 ***	3.188 **	2.25 *
b 'v' d	8.19 ***	8.92 ***	6.87***

$p \leq 0.05$ * $p \leq 0.01$ ** $p \leq 0.001$ ***

a 'v' b *t* values for probability are calculated from the mean of 8 and 12 determinations ± SD, with 18 *degrees of freedom.*

c 'v' d *t* values for probability are calculated from the mean of 9 and 12 determinations ± SD, with 19 *degrees of freedom.*

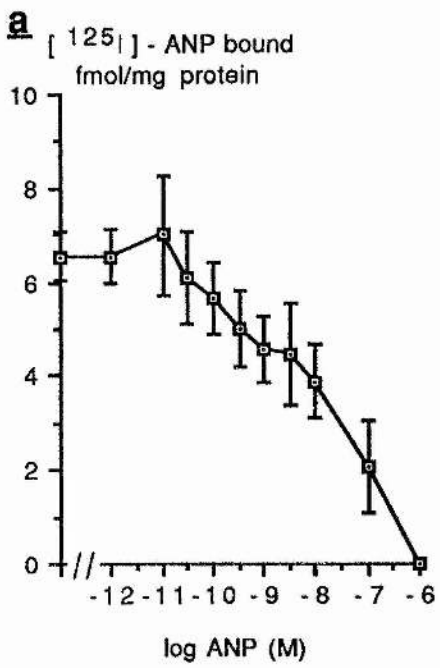
a 'v' c *t* values for probability are calculated from the mean of 8 and 9 determinations ± SD, with 15 *degrees of freedom.*

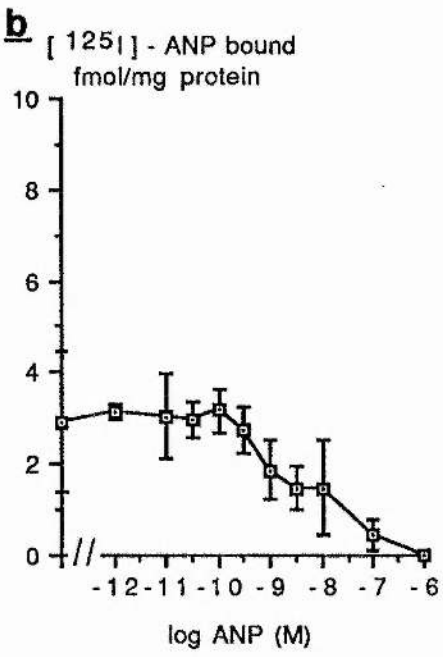
b 'v' d *t* values for probability are calculated from the mean of 12 determinations ± SD, with 22 *degrees of freedom.*

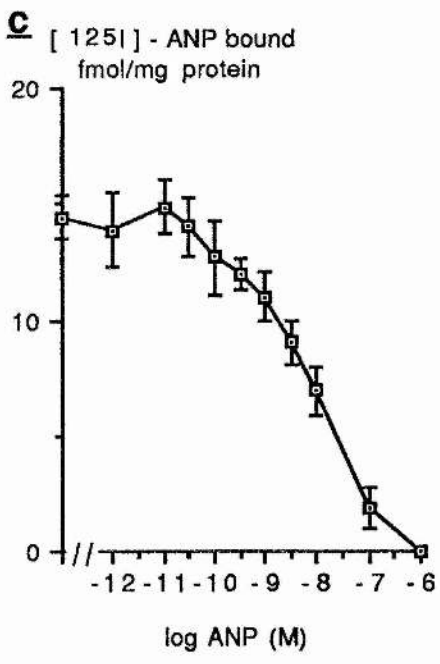
Figure 4.2

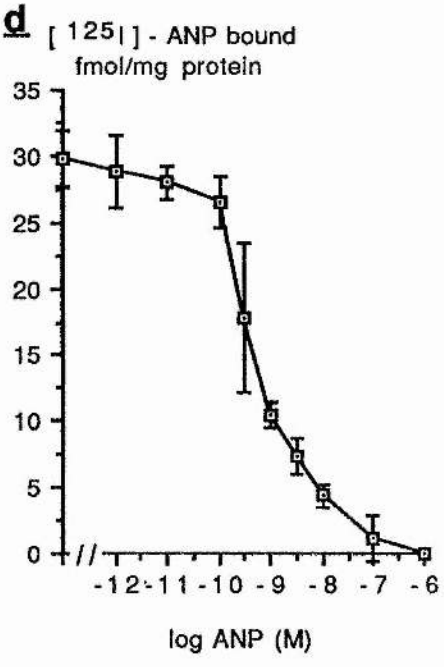
Inhibition of [^{125}I]-ANP binding in Dahl Rat Liver Plasma Membranes by ANP.

Dose response curves for ANP displacement of [^{125}I]-ANP binding to rat liver plasma membranes (25 μg protein/100 μl). In each graph the results shown are corrected for NSB and are mean experimental values from three individual membrane preparations. Experiments were conducted in the presence of 50 pM [^{125}I]-ANP for (a) Dahl-R rats on a 0.8% NaCl diet, (b) Dahl-R rats on an 8% NaCl diet, (c) Dahl-S rats on a 0.8% NaCl diet and (d) Dahl-S rats on an 8% NaCl diet. Each point is the mean of at least 9 determinations. Phosphoramidon and PMSF concentrations in the incubation buffer were 0.1 μM and 0.1 mM respectively.


a
[125I] - ANP bound
fmol/mg protein
10
8
6
4
2
0
-12 -11 -10 -9 -8 -7 -6
log ANP (M)


b
[125I] - ANP bound
fmol/mg protein
10
8
6
4
2
0
-12 -11 -10 -9 -8 -7 -6
log ANP (M)


c
[125I] - ANP bound
fmol/mg protein
20
10
0
-12 -11 -10 -9 -8 -7 -6
log ANP (M)


d
[125I] - ANP bound
fmol/mg protein
35
30
25
20
15
10
5
0
-12 -11 -10 -9 -8 -7 -6
log ANP (M)

experiments were combined. Scatchard analysis of the data (see fig. 4.3) resulted in a linear plot for group (d) only with K_d and B_{max} values of 412 ± 67 pM and 282 ± 34.4 fmol/mg protein. No statistically significant Scatchard plot could be calculated for groups (a), (b) and (c). Results in the absence of unlabelled ANP also indicated low levels of [^{125}I]-ANP binding (between 3 and 7 fmol [^{125}I]-ANP bound/mg protein) in the membrane fractions isolated from groups (a) and (b). To check if ANP was being broken down under the experimental conditions used the concentrations of phosphoramidon and PMSF were subsequently increased 10 - fold in the incubation buffer to 1 μM and 1 mM respectively. The previous experiments with each group of rats were repeated (see fig. 4.4). Competitive displacement binding analysis revealed that [^{125}I]-ANP specific binding in the presence of 50 pM [^{125}I]-ANP was increased by 2.7, 6.2, 1.4 and 1-fold for groups (a), (b), (c) and (d) respectively (see fig. 4.5). Scatchard analysis of these data (see fig. 4.6) resulted in linear plots for groups (a), (b), (c) and (d) with K_d and B_{max} values of 245 ± 80 pM and 104 ± 10 fmol/mg protein, 258 ± 18 pM and 120 ± 28 fmol/mg protein, 285 ± 32 pM and 148 ± 20 fmol/mg protein, 288 ± 84 pM and 217 ± 40 fmol/mg protein respectively (see table 4.3). Scatchard analysis revealed linear plots for membrane fractions from each group of rats suggesting the presence of only one affinity class of [^{125}I]-ANP receptor site. The ring deleted analogue des [QSGLG] ANP (4-23)-NH_2, which has been shown to be specific for the ANP-C receptor was used to displace [^{125}I]-ANP from rat liver plasma membranes (see fig. 4.7). Results obtained showed that 0.01μM des-ANP was able to displace 33.6 ± 6.5%, 26.5 ± 13.1%, 26.9 ± 7.1% and 13.7 ± 7.5% of the total receptor sites from liver plasma membranes of rat groups (a), (b), (c) and (d) respectively, indicating the percentage of ANP-C receptors present in these membrane preparations.

Figure 4.3

Scatchard plots of the data shown in Figure 4.2.

In each graph the results shown are corrected for NSB and are mean experimental values from three individual membrane preparations. Experiments were conducted in the presence of 50 pM [^{125}I]-ANP for (a) Dahl-R rats on a 0.8% NaCl diet, (b) Dahl-R rats on an 8% NaCl diet, (c) Dahl-S rats on a 0.8% NaCl diet and (d) Dahl-S rats on an 8% NaCl diet. Each point is the mean of at least 3 determinations.

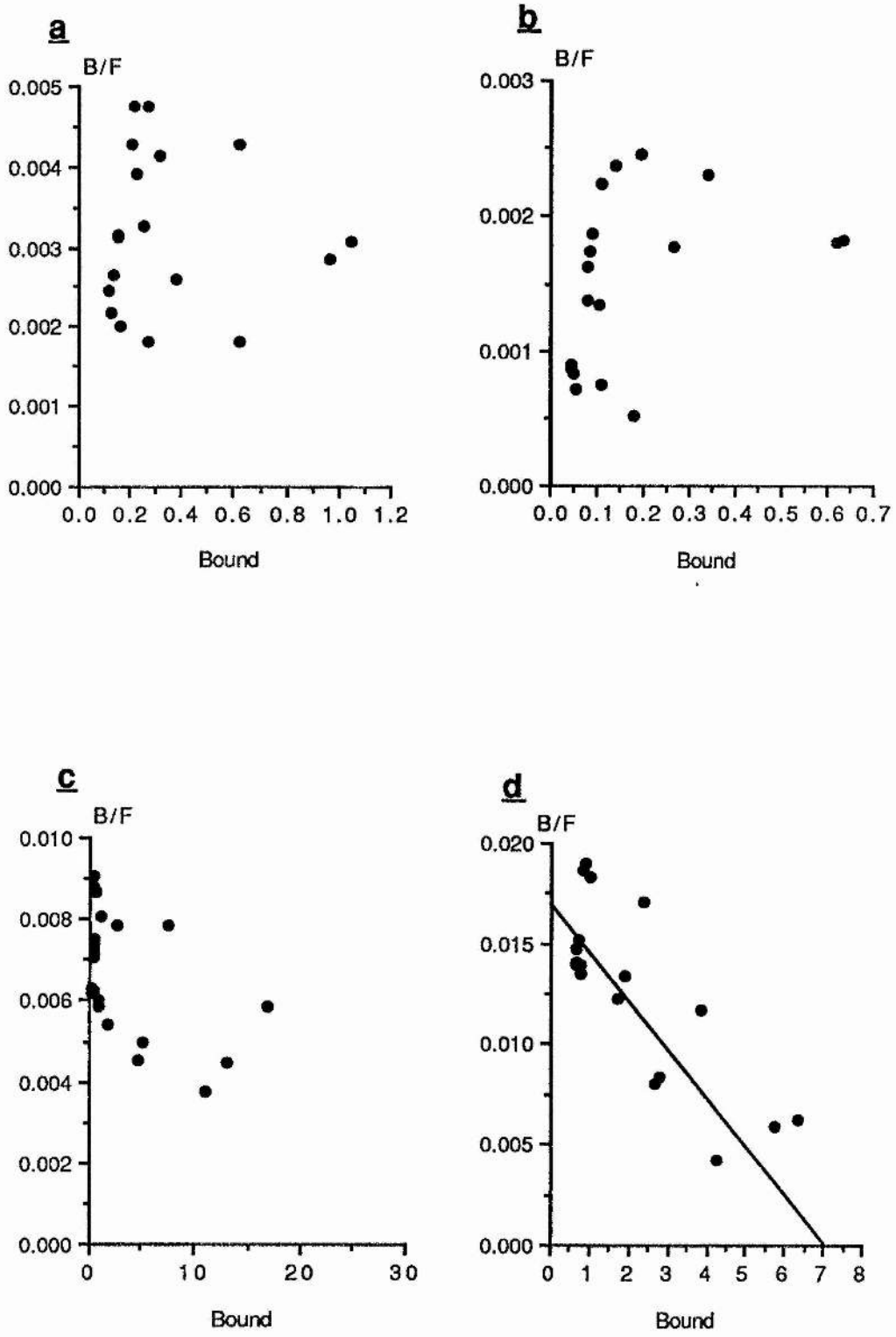

a
B/F
0.005
0.004
0.003
0.002
0.001
0.000
0.0 0.2 0.4 0.6 0.8 1.0 1.2
Bound
b
B/F
0.003
0.002
0.001
0.000
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7
Bound
c
B/F
0.010
0.008
0.006
0.004
0.002
0.000
0 10 20 30
Bound
d
B/F
0.020
0.015
0.010
0.005
0.000
0 1 2 3 4 5 6 7 8
Bound

Figure 4.4

Inhibition of [^{125}I]-ANP binding in Dahl-rat liver plasma membranes by ANP.

Dose response curves for ANP displacement of [^{125}I]-ANP binding to rat liver plasma membranes (25 μg protein/100 μl). In each graph the results shown are corrected for NSB and are mean experimental values from three individual membrane preparations. Experiments were conducted in the presence of 50 pM [^{125}I]-ANP for (a) Dahl-R rats on a 0.8% NaCl diet, (b) Dahl-R rats on an 8% NaCl diet, (c) Dahl-S rats on a 0.8% NaCl diet and (d) Dahl-S rats on an 8% NaCl diet. Each point is the mean of at least 9 determinations. Phosphoramidon and PMSF concentrations in the incubation buffer were 1 μM and 1 mM respectively.

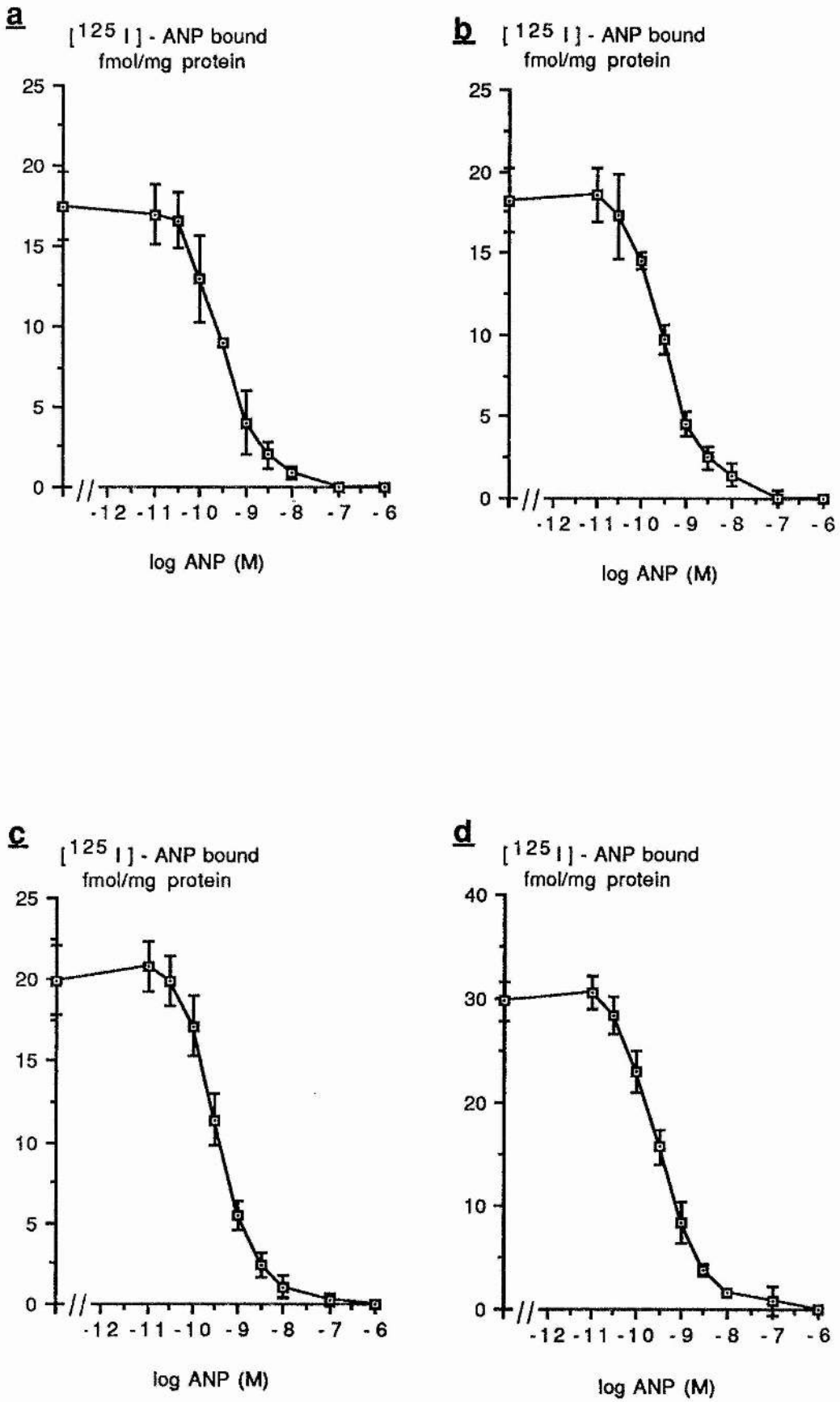

a
[125 I] - ANP bound
fmol/mg protein
25
20
15
10
5
0
-12 -11 -10 -9 -8 -7 -6
log ANP (M)
b
[125 I] - ANP bound
fmol/mg protein
25
20
15
10
5
0
-12 -11 -10 -9 -8 -7 -6
log ANP (M)
c
[125 I] - ANP bound
fmol/mg protein
25
20
15
10
5
0
-12 -11 -10 -9 -8 -7 -6
log ANP (M)
d
[125 I] - ANP bound
fmol/mg protein
40
30
20
10
0
-12 -11 -10 -9 -8 -7 -6
log ANP (M)

Figure 4.5

The effect of Phosphoramidon and PMSF on [^{125}I]-ANP binding in Dahl Rat liver plasma membranes.

The results shown are corrected for NSB and are mean experimental values from three individual membrane preparations. Experiments were conducted in the presence of 50 pM [^{125}I]-ANP and results expressed as [^{125}I]-ANP bound/fmol/mg protein for (a) Dahl-R rats on a 0.8% NaCl diet, (b) Dahl-R rats on an 8% NaCl diet, (c) Dahl-S rats on a 0.8% NaCl diet and (d) Dahl-S rats on an 8% NaCl diet. Each point is the mean of at least 9 determinations.

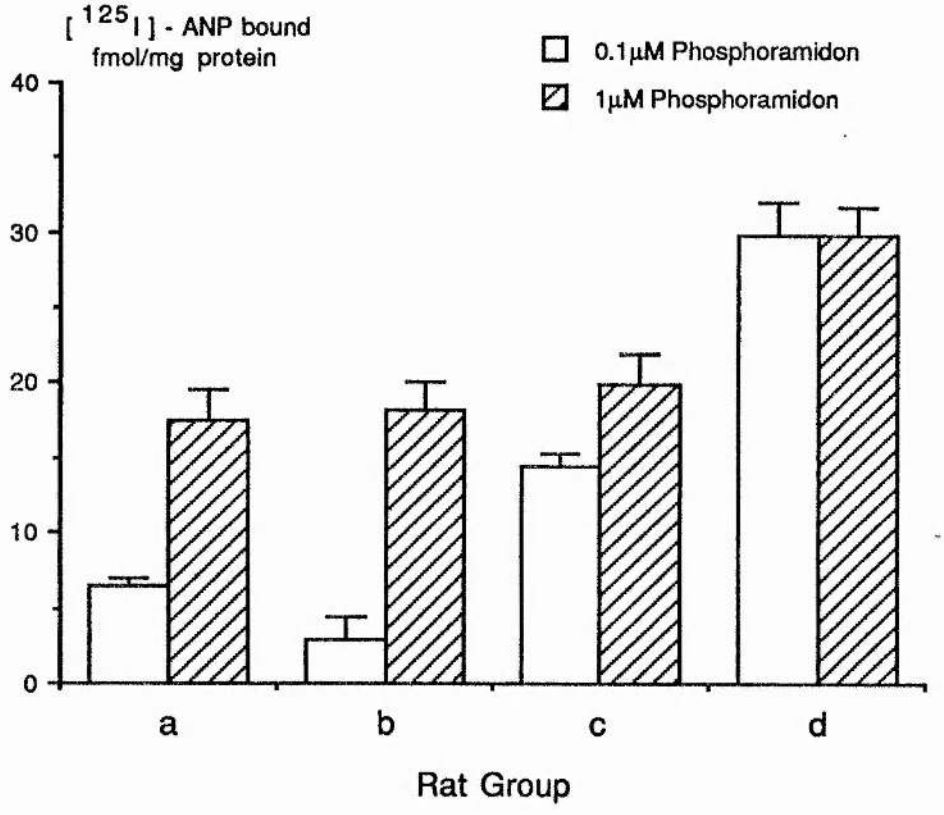


Figure 4.6

Scatchard plots of the data shown in Figure 4.4.

The results shown, corrected for NSB are for three individual experiments in the presence of 50 pM ^{125}I-ANP for (a) Dahl-R rats on a 0.8% NaCl diet, (b) Dahl-R rats on an 8% NaCl diet, (c) Dahl-S rats on a 0.8% NaCl diet and (d) Dahl-S rats on an 8% NaCl diet. Each point is the mean of at least 3 determinations.

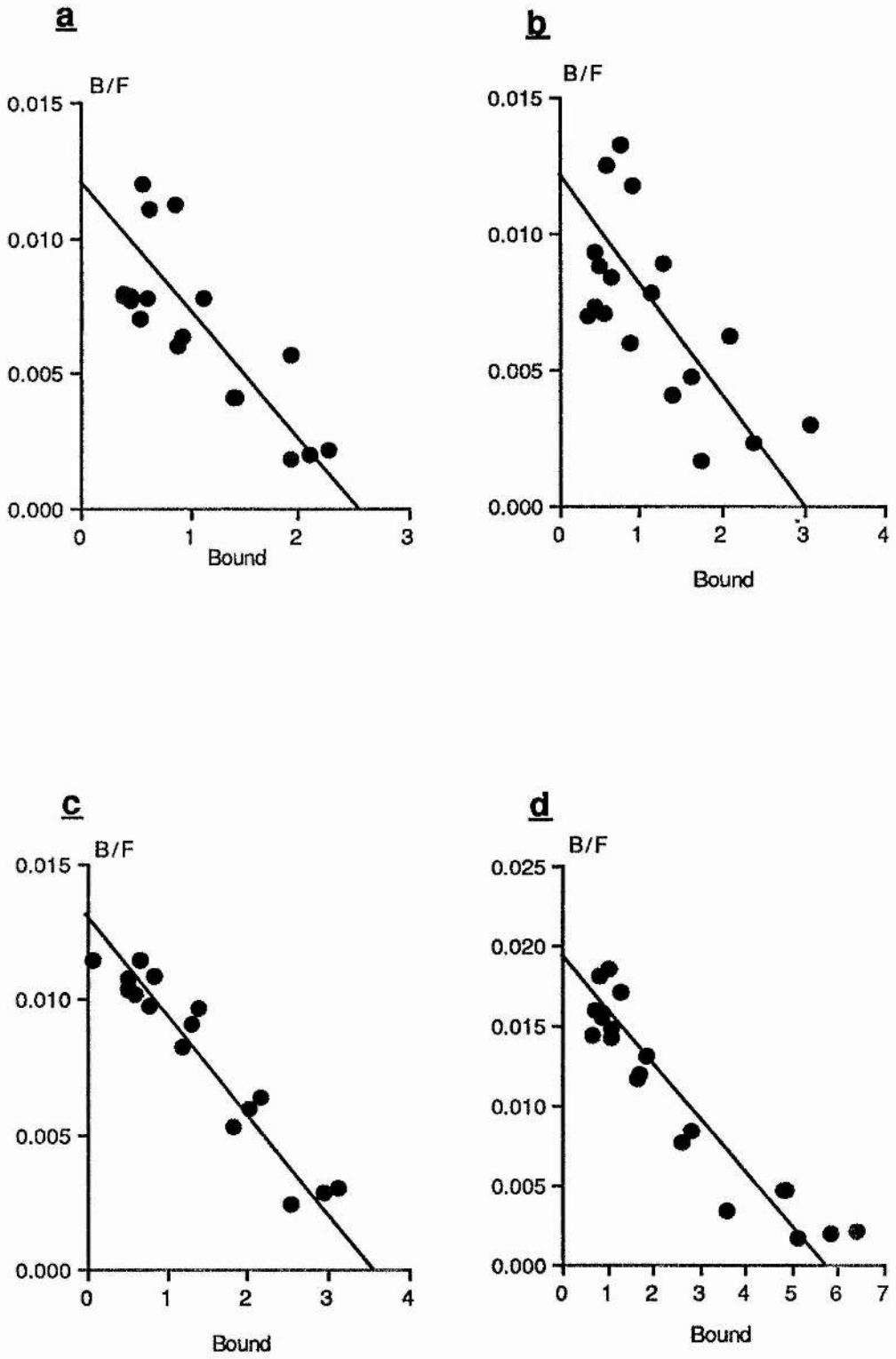

a
B/F
0.015
0.010
0.005
0.000
0
1
2
3
Bound
b
B/F
0.015
0.010
0.005
0.000
0
1
2
3
4
Bound
c
B/F
0.015
0.010
0.005
0.000
0
1
2
3
4
Bound
d
B/F
0.025
0.020
0.015
0.010
0.005
0.000
0
1
2
3
4
5
6
7
Bound

Table 4.3

Comparison of the effects of Phosphoramidon and PMSF on ^{125}I-ANP binding kinetics in Dahl-Rat liver membranes.

The K_d and B_{max} values for (a) Dahl-R rats on a 0.8% NaCl diet, (b) Dahl-R rats on an 8% NaCl diet, (c) Dahl-S rats on a 0.8% NaCl diet and (d) Dahl-S rats on an 8% NaCl diet, were calculated from Scatchard analysis. Each value is the mean of at least 3 experiments ± S.D.

	0.1μM Phph'don	0.1mM PMSF	1μM Phph'don	1mM PMSF
RAT GROUP	K_d (pM)	B_{max} (fmol/mg)	K_d (pM)	B_{max} (fmol/mg)
a	NC	NC	245 ± 80	104 ± 10
b	NC	NC	258 ± 18	120 ± 28
c	NC	NC	285 ± 32	148 ± 20*
d	412 ± 67	282 ± 34.4	288 ± 84	217 ± 40**

Phph'don; Phosphoramidon

PMSF; Phenyl-methyl-sulphonyl-fluoride

NC; Not Calculated

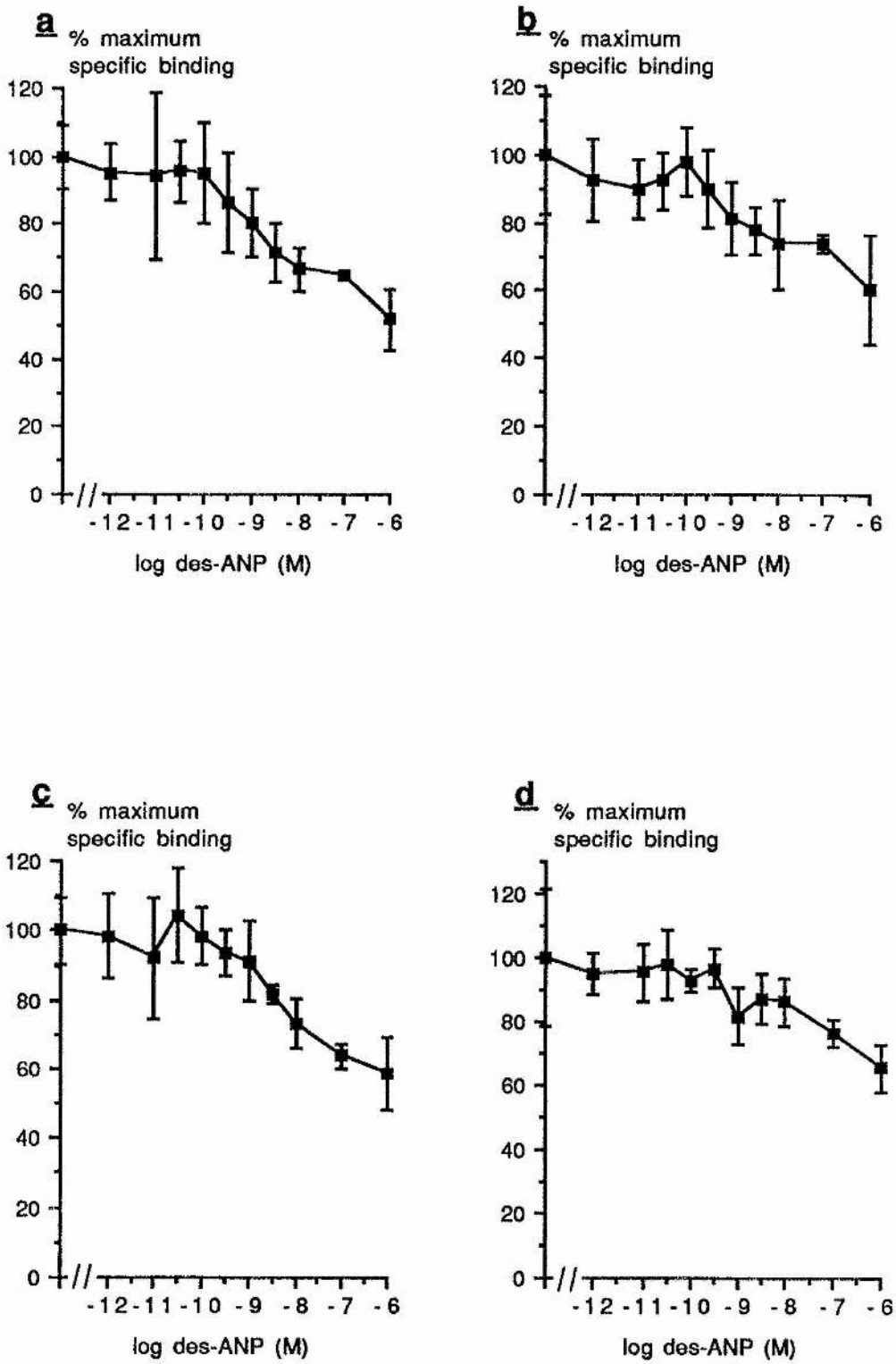
* Dahl-S rat on 0.8% NaCl diet significantly different from Dahl-R rat on an equivalent diet ($p \leq 0.05$ mean of 3 experiments ± SD)

** Dahl-S rat on an 8% NaCl diet significantly different from Dahl-R rat on an equivalent diet ($p \leq 0.05$ mean of 3 experiments ± SD)

Figure 4.7

Inhibition of [^{125}I]-ANP binding in Dahl-rat liver plasma membranes by des [QSGLG] ANP (4-23) - NH_2.

Dose response curves for des [QSGLG] ANP(4-23)-NH_2 competitive displacement of [^{125}I]-ANP binding to rat liver plasma membranes (25 μg protein/100 μl). In each graph the results shown are corrected for NSB and are mean experimental values from three individual membrane preparations. Experiments were conducted in the presence of 50 pM [^{125}I]-ANP for (a) Dahl-R rats on a 0.8% NaCl diet, (b) Dahl-R rats on an 8% NaCl diet, (c) Dahl-S rats on a 0.8% NaCl diet and (d) Dahl-S rats on an 8% NaCl diet. Each point is the mean of at least 9 determinations.


a
% maximum
specific binding
120
100
80
60
40
20
0
-12 -11 -10 -9 -8 -7 -6
log des-ANP (M)
b
% maximum
specific binding
120
100
80
60
40
20
0
-12 -11 -10 -9 -8 -7 -6
log des-ANP (M)
c
% maximum
specific binding
120
100
80
60
40
20
0
-12 -11 -10 -9 -8 -7 -6
log des-ANP (M)
d
% maximum
specific binding
120
100
80
60
40
20
0
-12 -11 -10 -9 -8 -7 -6
log des-ANP (M)

4.3.2 [^{125}I]-ANP receptor binding to Wistar rat liver plasma membranes

Displacement of 50 pM [^{125}I]-ANP from liver plasma membranes isolated from male Wistar rat was measured in two experiments from one preparation. Incubations were for 1 h at room temperature with increasing concentrations of ANP and in the presence of 1 μM phosphoramidon and 1 mM PMSF. Results are expressed as [^{125}I]-ANP bound (fmol/mg protein) and the results of the two individual experiments were combined, (see fig. 4.8 a). Scatchard analysis of this data (see fig. 4.8 b), resulted in a linear plot with K_d and B_{max} values of 332 ± 79 pM and 56 ± 8 fmol/mg protein respectively, suggesting the presence of only one affinity receptor class. The ANP analogue des-ANP was then used as a competitve ligand to displace [^{125}I]-ANP from the plasma membranes (see fig. 4.8c). The resulting dose response curve indicated that 0.1 μM des-ANP was able to displace 89% of the total receptor sites from Wistar rat liver plasma membranes, indicating that this is the percentage of the total receptor density of ANP-C receptors present in these membrane preparations.

4.4 Receptor Crosslinking Experiments.

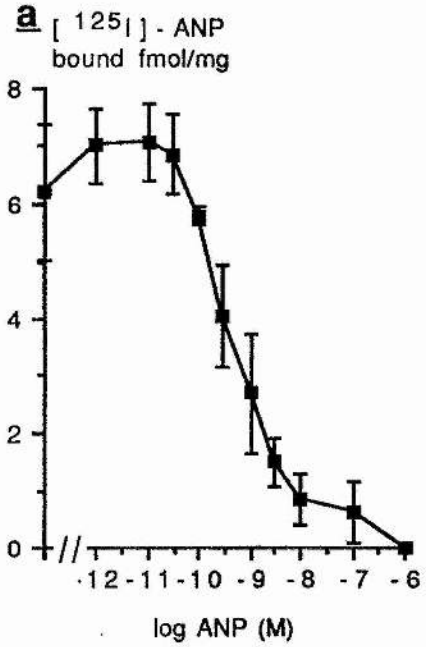
4.4.1 [^{125}I]-ANP crosslinking to Dahl rat liver plasma membranes.

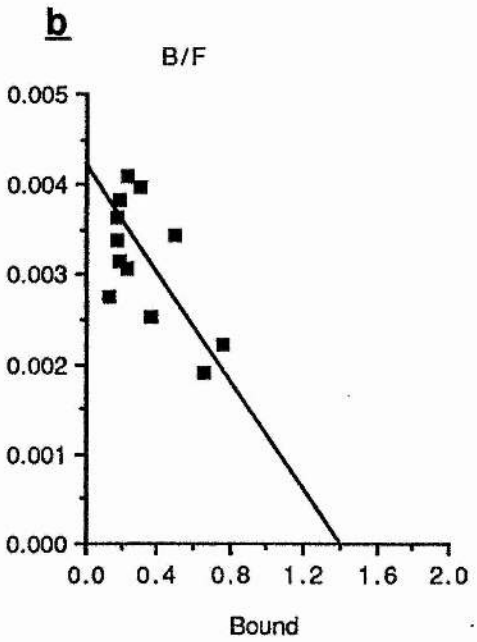
Since the binding analysis with ANP and des-ANP indicated an increased density of ANP receptors in the Dahl-Sensitive rat group on an 8% NaCl diet, crosslinking experiments were carried out with purified plasma membranes isolated from Dahl-S rats livers. However, DSS (0.1 mM) proved to be unsuccessful at crosslinking [^{125}I]-ANP to receptors in this preparation. The crosslinking protocol was altered several times to optimise the conditions. Alterations included increasing the concentration of DSS to 0.5 mM and also changing the crosslinker to ethylene glycol *bis* (succinimidylsuccinate) (EGS), which is 16.1 A in length

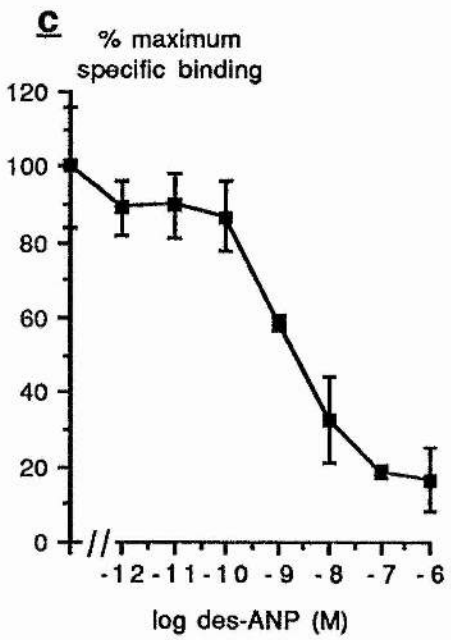
Figure 4.8

Results of the displacement of [^{125}I]-ANP in Wistar rat liver membrane preparations by ANP and des-ANP.

In each graph the results shown are corrected for NSB and are mean experimental values for two individual experiments (one membrane preparation) in the presence of 50 pM [^{125}I]-ANP. (a) Dose response curve for ANP competitive displacement of [^{125}I]-ANP, (b) Scatchard analysis of data presented in (a) and (c) dose response curve for des-ANP competitive displacement of [^{125}I]-ANP. Each point is the mean of at least 6 determinations for (a) and (c) and at least 3 determinations for (b).


a
[125I] - ANP
bound fmol/mg
8
6
4
2
0
-12 -11 -10 -9 -8 -7 -6
log ANP (M)


b
B/F
0.005
0.004
0.003
0.002
0.001
0.000
0.0 0.4 0.8 1.2 1.6 2.0
Bound


c
% maximum
specific binding
120
100
80
60
40
20
0
-12 -11 -10 -9 -8 -7 -6
log des-ANP (M)

as opposed to DSS which is 11.4 A in length. Ethylene glycol *bis* (succinimidylsuccinate) was also used at a concentration of 0.5 mM. Under all conditions tested only DSS (0.5 mM) proved to be marginally successful at crosslinking [^{125}I]-ANP to two receptors in the purified liver plasma membranes with molecular weights of 60 kDa and 120 kDa (see fig. 4.9). Ethylene glycol *bis* (succinimidylsuccinate) (0.5 mM) had a limited amount of success and was found only to crosslink [^{125}I]-ANP to a protein of 60 kDa molecular weight (see fig. 4.9). The efficiency of crosslinking was extremely low and intensity of the bands did not reflect the results from ANP analogue binding analysis which had indicated the presence of a high density (86.3 ± 7.5%) of ANP-B receptors and a lower density (13.7 ± 7.5%) of ANP-C receptors.

4.4.2 [^{125}I]-ANP crosslinking to Wistar rat liver plasma membranes.

Crosslinking experiments with purified plasma membranes of Wistar rat liver were repeatedly unsuccessful. Initially, this was surprising since effective crosslinking was carried out using similar protocols in bovine sarcolemmal membrane preparations which had a similar receptor density to that of the Dahl rat liver membranes. The results obtained with purified plasma membranes of Wistar rat liver indicate that the crosslinker DSS (0.1 mM) is not suitable for the determination of receptor subtypes in this tissue. The photoaffinity crosslinker N-hydroxysuccinimidyl-4- azidobenzoate (HSAB) was also used with these membrane preparations and provided similar negative results.

4.5 Guanylate Cyclase Experiments.

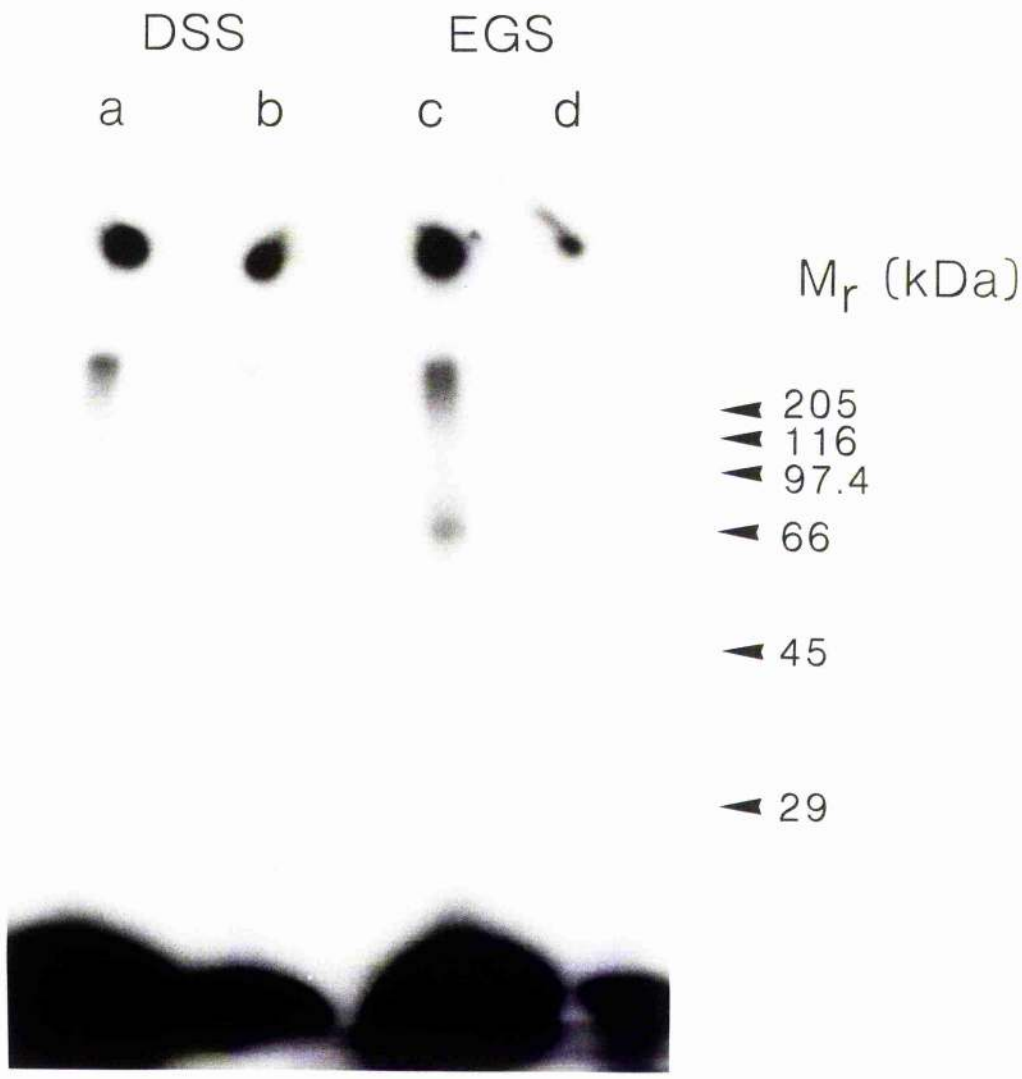
4.5.1 Guanylate cyclase activity in Dahl rat liver plasma membranes.

Atrial natriuretic peptide-stimulated dose dependent increases in guanylate cyclase activity were shown in purified rat liver plasma

Figure 4.9

Crosslinking of [^{125}I]-ANP to Dahl-rat liver plasma membrane homogenates at room temperature in the presence or absence of ANP

[^{125}I]-ANP (50 pM) was incubated with liver plasma membranes (700 μg) at room temperature, in the absence (lanes a and c) and in the presence (lanes b and d) of 0.1 μM ANP. Lanes a and b are in the presence of the crosslinker DSS and lanes c and d are in the presence of the crosslinker EGS. Migration of the molecular weight standards is indicated.


DSS
EGS
a
b
c
d
M_r (kDa)
205
116
97.4
66
45
29

membranes of rat groups (a), (b), (c) and (d) (see fig. 4.10). The assay was carried out as described in the Materials and Methods. Basal and ANP stimulated guanylate cyclase activities were compared for each group of rats (see tables 4.4, 4.5 and 4.6). These results indicated that groups (a), (b) and (c) had similar basal guanylate cyclase activities with values of 22 ± 4, 17 ± 5 and 27 ± 5 pmol cGMP/mg protein/min produced respectively. The Dahl-S rats on an 8% NaCl diet, (group d) had a significantly higher basal value of 44 ± 10 pmol cGMP/mg protein/min produced than all other three groups. The half maximum values required for stimulated guanylate cyclase activity for rat groups (a), (b), (c) and (d) were 3.16 ± 2.04 nM, 2.33 ± 0.47 nM, 2.9 ± 0.71 nM and 7.33 ± 2.05 nM respectively, (see tables 4.4 and 4.6). The Dahl-S rats on a high salt diet, (group d) had a significantly higher EC_{50} value than Dahl-R rats on an 8% NaCl diet (group b) and Dahl-S rats on a 0.8% NaCl diet (group c).

4.5.2 Guanylate cyclase activity in Wistar rat liver plasma membranes.

Small ANP-stimulated dose dependent increases in guanylate cyclase activity were shown in purified rat liver plasma membranes of the Wistar rat, (see fig. 4.11). Basal levels ranged from 44 to 54 pmol cGMP/mg protein/min produced with stimulated levels increasing less than 2-fold to 62 to 73 pmol cGMP/mg protein/min produced. This result indicated the presence of an extremely low density of ANP-B receptors with linked guanylate cyclase activity in Wistar rat liver.

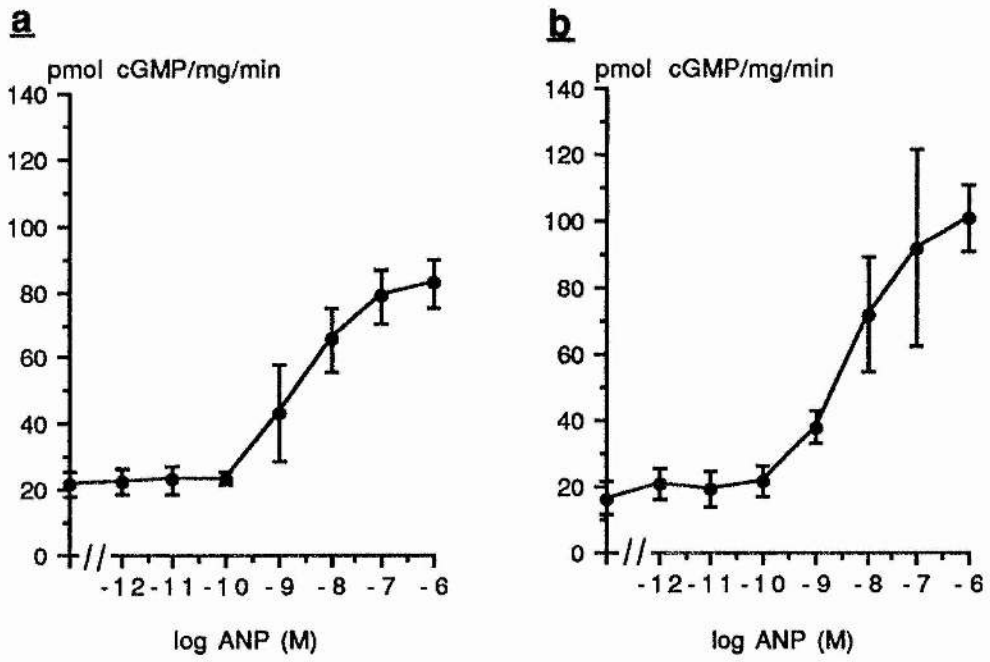
4.6 Discussion

The blood pressure results from the four groups of Dahl rats indicated that Dahl-S rats on a 0.8% NaCl diet have increased systolic, mean and diastolic blood pressures when compared to Dahl-R rats on the same diet. Dahl-S rats on an 8% NaCl diet have increased systolic, mean and diastolic blood

Figure 4.10

ANP stimulated cGMP production in Dahl-rat liver membranes.

Liver membranes (10 μg) were incubated at 37 °C for 20 min with increasing concentrations of ANP as described in the Materials and Methods. The results shown indicate ANP-stimulated guanylate cyclase activities in membranes isolated from (a) Dahl-R rats on a 0.8% NaCl diet, (b) Dahl-R rats on an 8% NaCl diet, (c) Dahl-S rats on a 0.8% NaCl diet and (d) Dahl-S rats on an 8% NaCl diet. Each point represents the mean ± S.D. of eighteen individual measurements taken from three separate liver preparations.


a
pmol cGMP/mg/min
140
120
100
80
60
40
20
0
-12 -11 -10 -9 -8 -7 -6
log ANP (M)
b
pmol cGMP/mg/min
140
120
100
80
60
40
20
0
-12 -11 -10 -9 -8 -7 -6
log ANP (M)

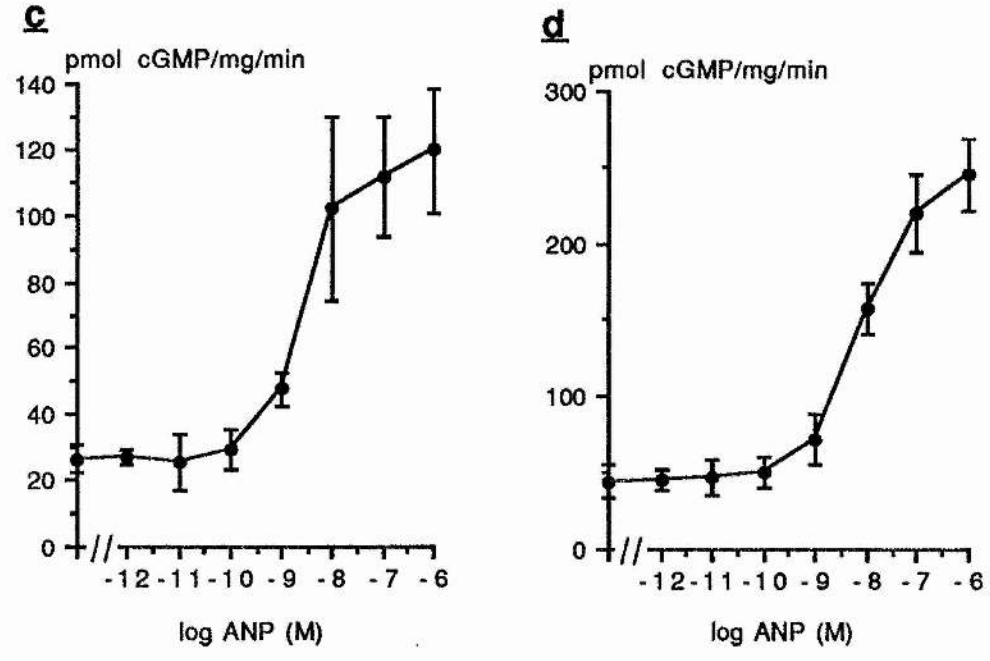

c
pmol cGMP/mg/min
140
120
100
80
60
40
20
0
-12 -11 -10 -9 -8 -7 -6
log ANP (M)
d
pmol cGMP/mg/min
300
200
100
0
-12 -11 -10 -9 -8 -7 -6
log ANP (M)

Table 4.4

Comparison of basal and ANP-stimulated cGMP production in male Dahl-R and Dahl-S rat liver membranes.

Basal and ANP-stimulated guanylate cyclase activities were calculated for liver membranes isolated from (a) Dahl-R rats on a 0.8% NaCl diet, (b) Dahl-R rats on an 8% NaCl diet, (c) Dahl-S rats on a 0.8% NaCl diet and (d) Dahl-S rats on an 8% NaCl diet. Each experimental value is the mean of 3 three separate liver preparations ± S.D.

pmol cGMP/mg protein/min

Rat Group	Basal	1μM ANP	Fold increase on basal
a	22 ± 4	83 ± 8	3.8
b	17 ± 5	101 ± 10	6.15
c	27 ± 5	119 ± 20*	4.49
d	44 ± 10** (*)	245 ± 24*** +	5.6

* significantly different from Dahl-R rats on a 0.8% NaCl diet ($p \leq 0.05$ mean of 3 experiments ± SD)

** significantly different from Dahl-R rats on an 8% NaCl diet ($p \leq 0.02$ mean of 3 experiments ± SD)

*** significantly different from Dahl-S rats on a 0.8% NaCl diet ($p \leq 0.01$ mean of 3 experiments ± SD)

+ significantly different from Dahl-R rats on an 8% NaCl diet ($p \leq 0.001$ mean of 3 experiments ± SD)

Figure 4.11

Production of cGMP in Wistar rat liver membranes by ANP.

Liver membranes (10 μg) were incubated at 37 °C for 20 min with increasing concentrations of ANP. Each point represents the mean ± S.D. of twelve individual measurements taken from two individual experiments from a single membrane preparation.

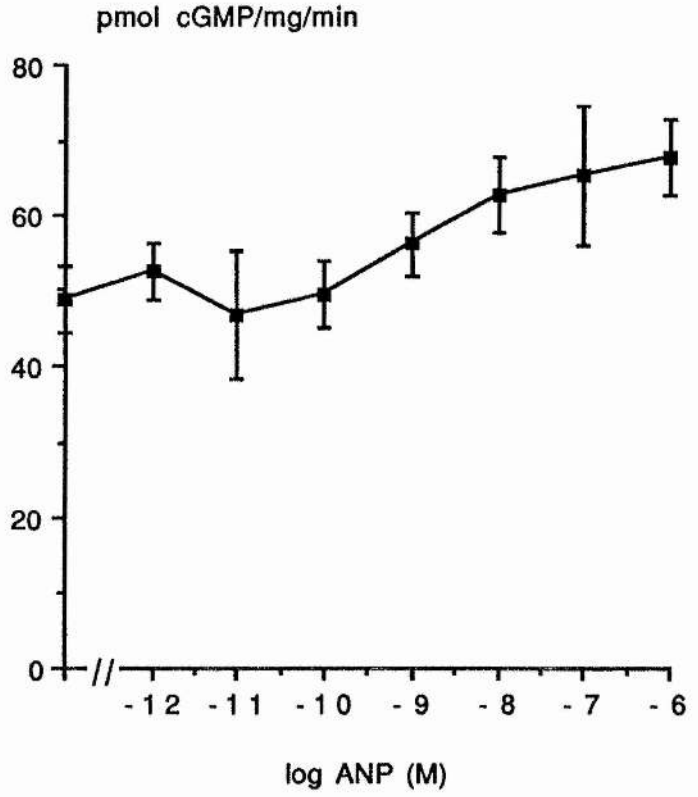


Table 4.5

Comparison of the concentration required for half maximal stimulation (EC_{50}) of cGMP production and the K_d values for [^{125}I]-ANP specific binding.

EC_{50} values were calculated for cGMP production and [^{125}I]-ANP specific binding in liver membranes isolated from (a) Dahl-R rats on a 0.8% NaCl diet, (b) Dahl-R rats on an 8% NaCl diet, (c) Dahl-S rats on a 0.8% NaCl diet and (d) Dahl-S rats on an 8% NaCl diet.

RAT GROUP	EC_{50}cGMP (nM)	K_d (pM)
a	3.16 ± 2.04	245 ± 80
b	2.33 ± 0.47	258 ± 18
c	2.9 ± 0.71	285 ± 32
d	7.33 ± 2.05* +	288 ± 84

* significantly different from the EC_{50} value of Dahl-S rats on a 0.8% NaCl diet ($p \leq 0.05$ mean of 3 experiments ± SD)

+ significantly different from the EC_{50} value of Dahl-R rats on an 8% NaCl diet ($p \leq 0.05$ mean of 3 experiments ± SD)

Table 4.6

***t* values for kinetic constants obtained for ANP binding and activation of guanylate cyclase in liver membranes isolated from male Dahl-R and Dahl-S rats.**

	t value				
Rat Groups	K_d	B_{max}	EC_{50} guanylate cyclase activity	Basal guanylate cyclase activity	ANP-stim'd max.guanylate cyclase activity
a 'v' b	0.27	0.932	0.74	1.352	2.434
c 'v' d	0.058	2.67	2.86*	2.63*	6.986***
a 'v' c	0.764	3.4*	0.237	1.352	2.894*
b 'v' d	0.604	3.44*	3.368*	4.183**	9.593****

* $p \leq 0.05$ ** $p \leq 0.02$ *** $p \leq 0.01$ **** $p \leq 0.001$

These *t* values for probability are calculated from the mean of 3 experiments ± SD, with 4 *degrees of freedom.*

pressures when compared to Dahl-S and Dahl-R rats on a 0.8% and an 8% NaCl diet respectively. These results are in agreement with Snajdar & Rapp (1985) where differences in blood pressures were noted between Dahl-S and Dahl-R rats, however alterations in salt diet from, normal (1%) to low (0.3%) or high (8%) NaCl induced no change in blood pressure in the Dahl-S rats used in the study. The results presented are also in agreement with both Gutkwoska *et al.* (1986) and Schwartz *et al.* (1986) who reported that Dahl-S rats fed for 5 weeks on an 8% NaCl diet had increased blood pressure compared to Dahl-R rats on the same diet.

Results from [^{125}I]-ANP binding studies carried out with different phosphoramidon and PMSF concentrations indicated that 0.1 μM phosphoramidon and 0.1 mM PMSF were not sufficient to prevent the enzymatic degradation of ANP in groups (a) and (b) (the Dahl-R rats) and resulted in low levels (3 to 7 fmol [^{125}I]-ANP bound/mg protein) of [^{125}I]-ANP-specific binding. However increasing the inhibitor concentrations 10-fold to 1 μM phosphoramidon and 1 mM PMSF increased [^{125}I]-ANP-specific binding by 2.7 and 6.2-fold for groups (a) and (b) respectively and by 1.4 and 1-fold for groups (c) and (d) respectively. These results suggested that there maybe low concentrations of an ANP peptidase in groups (c) and (d) the Dahl-S rats and higher concentrations in groups (a) and (b) the Dahl-R rats. The increased concentrations of phosphoramidon and PMSF with groups (a) and (b) therefore were more effective at inhibiting the elevated peptidase activity, with the result of increased [^{125}I]-ANP receptor binding. (This observation is however only speculative and further experimental analysis on the relative concentrations of ANP peptidase activity in each group of rats is required). Results from the des-ANP binding analysis indicate that approximately 13-34% of the total ANP receptor numbers in each group of Dahl rat liver membranes were ANP-C

receptors (B_{max} of approximately 34.3 ± 3.3 fmol/mg, 31.8 ± 7.6 fmol/mg, 40 ± 5.4 fmol/mg and 30 ± 5.5 fmol/mg for groups (a), (b), (c) and (d) respectively). These results suggest that the majority (67-86%), (B_{max} of approximately 69.7 ± 3.3 fmol/mg, 88.2 ± 7.6 fmol/mg, 108 ± 5.4 fmol/mg and 187 ± 5.5 fmol/mg for groups (a), (b), (c) and (d) respectively) of the receptor sites in Dahl-S and Dahl-R rat liver plasma membranes are ANP-B receptors and that group (d), the sensitive rats on an 8% NaCl diet, possess an increased density of ANP-B receptors (with no change in affinity) when compared to the other three groups. Group (d) also has a significantly ($p \leq 0.05$) reduced density of ANP-C receptors when compared to group (c) the sensitive rats on a 0.8% NaCl diet. Unfortunately crosslinking experiments were unable to confirm these indications. Disuccinimydyl suberate (DSS) (0.1 mM) was unable to covalently attach [^{125}I]-ANP to any receptor subtypes in liver membranes. However, DSS (0.5 mM) proved to be marginally successful at crosslinking [^{125}I]-ANP to two receptors (with molecular weights of 60 kDa and 120 kDa) in purified liver plasma membranes isolated from Dahl-S rats. Ethylene glycol *bis* (succinimidylsuccinate) (EGS) at a concentration of 0.5 mM had a limited amount of success and was found only to covalently attach [^{125}I]-ANP to a protein of 60 kDa molecular weight. These results are perhaps due to the low efficiency of crosslinking to receptors in this tissue compared to that found in bovine ventricular sarcolemmal membrane preparations. The reason for this difference in crosslinking efficiency between the two membrane preparations remains unknown.

Results from guanylate cyclase experiments do indicate that liver membranes from sensitive rats on an 8% NaCl diet, (group d) have a significantly higher basal guanylate cyclase activity when compared to membranes isolated from all other groups. ANP-stimulated guanylate

cyclase activity is significantly higher in membranes isolated from sensitive rats on a 0.8% NaCl diet (group c), when compared to membranes isolated from resistant rats on the same diet (group a). In addition ANP-stimulated guanylate cyclase activity is significantly higher in membranes isolated from sensitive rats on an 8% NaCl diet, (group d) when compared to membranes isolated from sensitive rats on a 0.8% NaCl diet (group c) and membranes isolated from resistant rats on an 8% NaCl diet (group b). These results are in agreement with the results from the radio-receptor assay and suggest an increase in the population of ANP-B receptors with associated guanylate cyclase activity in membranes isolated from sensitive animals and more specifically from sensitive animals on an 8% NaCl diet. There is a poor correlation between ANP ligand binding and the subsequent stimulation of guanylate cyclase. The K_d values obtained for ANP ligand binding were found to be approximately 14, 9, 11 and 23-fold lower than the EC_{50} values required for ANP stimulated activation of guanylate cyclase for rat groups (a), (b), (c) and (d) respectively. These results, like the results found with bovine ventricular membrane preparations suggest a complex relationship between ANP ligand binding and the subsequent activation of guanylate cyclase.

Radio-receptor binding analysis of Wistar rat liver membranes indicated the presence of lower numbers of ANP-specific receptors than that found in any of the Dahl rat preparations. Results also indicated that the majority (greater than 90%) of receptors present in Wistar rat liver membranes were most likely ANP-C receptors. This result is contrary to the results obtained with the Dahl rats.

Experiments described demonstrate the presence of [^{125}I]-ANP receptors in partially purified plasma membrane preparations of whole rat liver. In addition, ANP-stimulated guanylate cyclase activity in liver membrane preparations was demonstrated. Results obtained also provide biochemical evidence for the presence of at least two ANP-specific receptors in the rat liver. ANP-specific receptors were identified as having similar molecular weights and biochemical characteristics as ANP receptors identified in other tissues and cells, (see section 1.2.2). Receptor binding/crosslinking and guanylate cyclase results are in agreement with Yip *et al.* (1989), where an ANP-specific receptor protein (140 kDa) in rat liver plasma membranes was identified and with Waldman *et al.* (1984) who showed guanylate cyclase activity in rat liver membrane preparations. However these results disagree with Wilcox *et al.* (1991) who utilised the technique of *in situ* hybridisation and were unable to identify ANP-specific (ANP-BR1, ANP-BR2 and ANP-C receptor) mRNA in rhesus monkey liver tissue.

The results described show a difference in receptor population between Wistar rat liver and Dahl rat liver. This would suggest that there is a genetic difference between these two rat models with regards to their ANP receptor populations in the liver. The physiological significance of ANP-B receptors in the Dahl rat liver, ANP-C receptors in the Wistar rat liver and the lack of ANP-specific mRNA expression in the rhesus monkey liver (Wilcox *et al.* 1991) remains to be elucidated.

The pathogenesis of sodium retention in hepatic cirrhosis has not been fully explained. The discovery of ANP invited speculation that a deficiency in the release of ANP or resistance to its actions in the cirrhotic state may occur (Warner *et al.* 1989). Epstein & Loutzenhiser (1989) have

suggested that the sodium retention associated with hepatic cirrhosis may be related in part to a reduced renal responsiveness to ANP and not to reduced ANP levels or to altered molecular species of ANP. The results presented here indicate the presence of specific ANP receptors in whole rat liver and also alterations in receptor numbers with a hypertensive state. It could therefore be hypothesised that there may be alterations in specific ANP receptor numbers in hepatic cirrhosis, in addition to a reduced renal responsiveness to ANP. Further experimental analysis is required to support this hypothesis.

In conclusion, the results presented in this thesis show, increased blood pressure, increased ANP-B receptor density and reduced ANP-C receptor density in the Dahl-S rat whole liver, with an 8% NaCl diet-induced hypertensive state. The role that this increase in ANP-B receptor density and reduction in ANP-C receptor density, in whole liver tissue plays in hypertension remains to be fully established. Recently, Kato *et al.* (1991) have shown in the bovine pulmonary artery endothelial cell line that an increase in intracellular cGMP resulting from activation of guanylate cyclase in the ANP-B receptor may cause the preferential down-regulation of the ANP-C receptor. This effect may regulate the rate of clearance of ANP from the circulation. Therefore it could be hypothesised that the increase in ANP-B receptors with ANP-specific binding resulting in increased guanylate cyclase activity and subsequent cGMP production, leads ultimately to increased vasorelaxtion and the possibility of decreasing BP and also to a down regulation of the ANP-C receptor population thus reducing the rate of clearance of ANP from the circulation. In parallel with this could be a decrease in ANP peptidase activity which in turn would mean reduced enzymatic degradation of ANP, thus enabling circulatory ANP concentrations to remain elevated

and able to bind to the increased ANP-B receptor population. This hypothesis does not explain why BP is highest in these Dahl-S rats. However, it is possible that BP would be even higher without the adjustments mentioned. Recent results from this laboratory (Adam *et al.* 1991 unpublished) have shown that plasma [ANP] in the Dahl-S rats used in this study ($n = 3$) are significantly ($p \leq 0.1$) increased (65.8 ± 17 fmol/ml and 59.3 ± 20 fmol/ml) when compared to Dahl-R rats (44.9 ± 16.4 and 38.4 ± 4.8 fmol/ml) on 0.8% and 8% NaCl diets respectively. These results are in agreement with Schwartz *et al.* (1986) and Gutkwoska *et al.* (1986) who investigated the effects of salt diet on BP and plasma [ANP] in Dahl rats. Both of these groups reported that Dahl-S rats fed on a high salt diet, (5 weeks on 8% salt) have a higher blood pressure and higher plasma [ANP] than Dahl-R rats, on the same diet. However these results are not in complete agreement with Snajdar & Rapp (1986) (see section 1.4.3.2). This group investigated Dahl-S and Dahl-R rats (2 month of age) on a normal (0.8%) salt diet and showed small differences in BP with no statistical differences in plasma [ANP]. In contrast to this, 6 month old Dahl-S rats showed increased BP and increased plasma [ANP] when compared to Dahl-R rats. Snajdar & Rapp (1986) also showed that changes of a similar order could be induced in young, 6 week old Dahl-S fed on a high (8%) salt diet for 3 weeks, compared to age-matched Dahl-R rats on the same diet. It would seem therefore that age and salt diet are both important determinants of increased plasma [ANP] and increased BP. Results from the Dahl rats (10 weeks old) used in this study perhaps only reflect a salt-induced rather than an age-induced increase in plasma [ANP] in the hypertensive state. It should also be noted that plasma [ANP]'s reported by Snajdar & Rapp (1986) for rats (2 months of age) expressed as pg/ml, ranging from 222 ± 22.2 pg/ml to 1079 ± 259.3 pg/ml (72.5 ± 7.25 fmol/ml to 352.3 ± 84.7 fmol/ml) are higher when compared to the results (ranging

from 38.4 ± 4.8 fmol/ml to 65.8 ± 17 fmol/ml) obtained in this laboratory for 10 week old rats. This discrepancy is perhaps due to differences in plasma sample collection and/or the radioimmunoassay protocol utilised. In 1989, Onwhochei and Rapp (1989) showed a deficiency in the ANP secretory mechanism of pre-hypertensive Dahl-S rats resulting in increased BP and decreased plasma [ANP]. It is possible that there is such a deficient mechanism present in the Dahl-S rats used in this study which at an early stage maintains the increase in BP and contributes to the hypertensive state. However the Dahl-S rats (10 week old) used in this study showed no indications of such defective mechanism, in that plasma [ANP] were increased compared to Dahl-R rats. Therefore, in the hypertensive state the Dahl-S rat has increased liver ANP-B receptors, decreased liver ANP-C receptors, increased plasma [ANP] and possibly a reduced ANP peptidase activity in the liver to counteract hypertension. The contribution of data from additional ANP-specific experiments in other Dahl rat tissues is necessary before the significance of the results of the Dahl rat liver can be fully interpreted. It is possible that hypertension in the Dahl rat induces both positive and negative (with regards to counteracting hypertension) alterations in ANP binding characteristics in various tissues. Further experimental studies (in various tissues) are being carried out in this laboratory to investigate, the binding characteristics of ANP and ANP receptor subtypes associated with the development of hypertension in the Dahl rat.

4.7 Summary

The above radio-receptor binding data indicate, that in plasma membranes isolated from Dahl rat liver the presence of two ANP-specific receptor subtypes. In this rat strain the ANP-C receptor is in the minority (less than 30% of the total ANP receptor population) and the ANP-B receptor is

in the majority. In the Dahl-S which is predisposed to the development of hypertension induced by a high salt diet shows an increase in blood pressure when fed on a normal (0.8% NaCl) diet, when compared to the Dahl-R rat on an equivalent diet. Access to a high salt dietary regime (8% NaCl) in the Dahl-S rat augments this condition. Analysis of receptor subtype in sensitive rats on an 8% NaCl diet indicate that ANP-B receptor density is higher than in the other three groups of rats, with increases in both basal and ANP stimulated guanylate cyclase activity. The indications from the studies in partially purified plasma membrane fractions of Wistar rat liver are of the presence of low densities of two ANP specific receptor subtypes. The ANP-B receptor being in the minority (less than 10% of the total receptor density) and the ANP-C receptor being in the majority. This is in direct contrast to the results found with the Dahl-rats. The precise physiological significance of ANP-B and ANP-C receptors in the Dahl and the the Wistar rat liver remains to be elucidated.

4.8 Future Perspectives

Atrial natriuretic peptide radioreceptor assays on isolated liver cell types such as hepatocytes, Kuppfer cells and pit cells would provide further information as to the precise location of ANP receptors in the liver. From the results presented ANP may play a regulatory role, perhaps in lipid and protein synthesis in the liver however this awaits investigation. Future experimental studies lie in refining receptor crosslinking assay techniques by expanding the range of crosslinkers and the experimental conditions utilised. Experimental studies on specific ANP peptidase concentrations in the liver also await investigation. Further studies are also required on the molecular biology of the ANP system to determine the precise nature of the ANP receptors present in the liver and to determine the physiological role of ANP in the liver.

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